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CAERULOMYCIN, A NEW ANTIBIOTIC FROM STREPTOMYCES CAERULEUS BALDACCI

I. PRODUCTION, ISOLATION, ASSAY, AND BIOLOGICAL PROPERTIES[1]

A. FUNK[2] AND P. V. DIVEKAR[3]

Abstract

Streptomyces caeruleus has been shown to produce a hitherto unknown antibiotic to which the name caerulomycin has been given. This was first obtained from a fresh isolate (PRL 1687), and subsequently, after the strain had been identified, from an authentic culture of *S. caeruleus*. It was extracted from culture filtrates with ether and purified as a colorless crystalline amphoteric substance, $C_{12}H_{11}O_2N_3$, which inhibited the growth of some filamentous fungi and yeasts and had weak activity against certain bacteria.

Introduction

During screening of *Streptomyces* species for production of antifungal substances, a contaminant capable of inhibiting various filamentous fungi and yeasts was isolated from one of the survey plates of Czapek solution agar. Shaken flask cultures of the organism grown in starch–Czapek medium showed similar activity and further investigation of the antibiotic was therefore undertaken. The organism was found to be a *Streptomyces* species, and has been identified by Dr. W. A. Taber of this laboratory as *Streptomyces caeruleus* Baldacci (2) as a result of a direct comparison of its morphology, cultural characteristics, and nutritional requirements with an authentic culture.

The antibiotic was isolated in a pure state from both PRL 1687 and the authentic culture of *S. caeruleus*[4] and has been studied in detail. Examination of its properties indicated that it was different from all known antibiotics and the name "caerulomycin" is therefore proposed for this substance. The

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Issued as N.R.C. No. 5180.
[2]Present address: Forest Biology Laboratory, Science Service, Victoria, British Columbia.
[3]National Research Council of Canada Postdoctorate Fellow, 1957–59.
[4]An authentic culture of *S. caeruleus* was obtained through the courtesy of Professor E. Baldacci, Istituto di Patologia Vegetale, Università degli studi, Milano, Italy, who has also informed the authors that he is not aware of any previous report on the production of antibiotics by this species.

[The previous number of Can. J. Microbiol. (**5**, 245–315) was issued June 2, 1959.]

present paper deals with methods for the production, isolation, and assay of caerulomycin and describes some of its physical and chemical properties. Further work on the characterization of both the antibiotic and the producing organism is in progress and will be reported at a later date.

Experimental

Culture

S. caeruleus PRL 1687 was maintained on Czapek solution agar plates. A dark navy-blue pigment diffused into the agar around the colonies and after about two weeks the agar was completely colored. A pH of 8 was found to be optimal for the growth (7).

Inoculum

Spores of *S. caeruleus* PRL 1687 were transferred to a flask of starch–Czapek medium and shaken at 30° C in the dark for 7 days on a rotary shaker describing a 2-in. circle and revolving at 110 r.p.m. The culture began to turn blue in 3 to 4 days and mycelial growth was in the form of pellets. For production of the antibiotic, 5 ml of this culture mixture was used to inoculate each 500-ml flask. Since the same fermentation conditions were used for production as for growing the spore inoculum, it was not necessary to start each run from a fresh spore inoculum. Several flasks saved from each production run were used as inoculum for the subsequent batch.

Medium

The composition of starch–Czapek medium used for both spore inoculum and antibiotic production was as follows: soluble starch, 20.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $NaNO_3$, 2.0 g; $CaCO_3$, 2.0 g; and distilled water, 1000 ml. One hundred milliliters volume was dispensed into each 500-ml conical flask and autoclaved for 15 minutes at 15 lb pressure.

Production

Cultures were incubated as described above and samples assayed at daily intervals. Maximum activity against *Candida albicans* was normally reached after 7 days when a concentration of 50 μg of caerulomycin per milliliter of culture was obtained. When sodium nitrate in the starch–Czapek medium was replaced by ammonium nitrate (0.2%) or ammonium succinate (0.2%), antibiotic production diminished though the growth appeared normal.

Assay

The antibiotic was conveniently assayed by the disk diffusion technique. For determining activity in the production cultures, a 3- to 4-ml sample was withdrawn aseptically at the required intervals, a paper disk dipped into the mixture was flicked free of excess liquid and immediately applied to a plate containing 10 ml of Waksman's Glucose Agar (WGA) (8) seeded with *C. albicans* at the rate of one 48-hour slant culture per 200 ml agar. The plates

were placed in the refrigerator for 1 hour (to allow the antibiotic to diffuse into the agar), incubated overnight at 30° C, and the inhibition zones measured and compared with those obtained from standard concentrations of the antibiotic.

Isolation of Caerulomycin

In a typical isolation procedure, 4 liters of active culture filtrate was extracted with three 1-liter portions of ether. No activity against the test organism, *C. albicans*, remained in the aqueous solution and the ethereal extract upon evaporation to dryness *in vacuo* gave a buff-colored amorphous powder (420 mg). This crude preparation was purified by one of the following methods: (*a*) after a preliminary wash with ice-cold petroleum ether (30–60° C), which removed some colored impurities and a small quantity of the antibiotic, it was crystallized twice from ethanol with the aid of animal charcoal to give pure caerulomycin as colorless needles, m.p. 175° C (yield from crude: 62%); or (*b*) it was sublimed in high vacuum at 120° C and the sublimate crystallized as before from ethanol (yield from crude: 60%).

Caerulomycin is soluble in ethyl acetate, acetone, ethanol, methanol, chloroform, and ether and sparingly soluble in benzene, petroleum ether, and water. It is insoluble in sodium hydrogen carbonate and sodium carbonate but readily dissolves in dilute solutions of sodium hydroxide and hydrochloric acid. An aqueous or alcoholic solution of the antibiotic gives a red color with ferric chloride but a more characteristic test for caerulomycin is the formation of a deep red color with ferrous salts in mineral acid solution. Such a reaction is typical of α,α'-dipyridyl (4).

The molecular formula, $C_{12}H_{11}O_2N_3$, has been assigned to caerulomycin on the basis of elementary analyses (found: C, 63.09; H, 4.99; N, 18.48%; calculated: C, 62.87; H, 4.84; N, 18.33%) and molecular weight values of 251 by the Rast method in camphor and 215 by the isothermal distillation method in acetone (3) (calculated for $C_{12}H_{11}O_2N_3$: 229). Kuhn-Roth estimation showed the absence of terminal methyl groups and no acetyl groups were detected by saponification. A Zeisel estimation indicated that one methoxy group was present (found: 13.15%; calculated: 13.54%) and one active hydrogen was found by a Zerewitinoff determination (found: 0.45%; calculated: 0.44%). By titration as a base against perchloric in acetic acid (6), neutralization equivalent values of 242 and 245 were obtained. As an acid, titrated against potassium methoxide in dimethyl formamide (5), a value of 227 was obtained. By a spectrophotometric procedure (1), the pK_a and pK_b were found to be 4.38 and 9.81 respectively. The infrared and ultraviolet absorption spectra of caerulomycin are shown in Figs. 1 and 2.

The antibiotic isolated in the same way from the authentic culture of *S. caeruleus* melted at 175° C, did not depress the melting point of caerulomycin isolated from PRL 1687, and had identical infrared and ultraviolet absorption spectra.

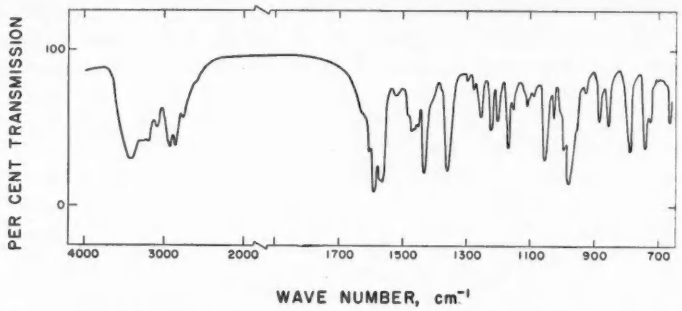

FIG. 1. Infrared absorption spectrum of caerulomycin (pressed potassium bromide pellet).

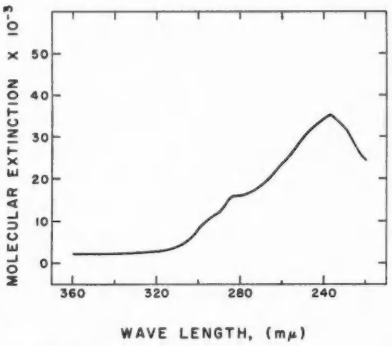

FIG. 2. Ultraviolet absorption spectrum of caerulomycin in ethanol.

Antimicrobial Properties of Caerulomycin

Crystalline caerulomycin was dissolved in 70% ethanol diluted with distilled water and dispensed into sterile petri plates. Ten milliliters of melted WGA medium were then poured into each plate and mixed well with the antibiotic solution. Concentrations of the antibiotic used were 0.1, 0.25, 0.5, 1.0, 5.0, 7.5, 10.0, 25.0, 50.0, 75.0, 100.0, 250.0, and 500.0 μg per milliliter of agar. Plates containing solvent alone were used as controls. The spores and cell suspensions of the test microorganisms were streaked on the plate with a wire loop. The plates were then incubated at 28° C and observed every 24 hours for 4 days. In testing the effect of caerulomycin on the germination of uredospores of *Puccinia graminis* var. *tritici* race 15B, one drop of 0.1% neomycin solution was added to the WGA plates to suppress bacterial growth. Suitable controls were also included. The uredospores were spread across the surface of the agar and examined for germination at daily intervals as above. The results of the antimicrobial tests are given in Table I.

TABLE I

Antimicrobial spectra of caerulomycin by agar dilution method

PRL No.	Name	Lowest concentration (μg/ml agar) for complete inhibition			
		24 hours	48 hours	72 hours	96 hours
1443 (ATCC 10123)	*Candida albicans*	5	10	10	10
1680	*Saccharomyces cerevisiae*	5	10	10	10
1452	*Isaria cretacea*	5	5	5	7.5
24	*Aspergillus niger*	10	75	75	75
1482	*Rhizopus nigricans*	5	5	7.5	10
1092	*Ustilago maydis*	10	15	15	15
	Puccinia graminis var. *tritici* race 15B (uredospore germination*)	100	100	100	100
B44	*Bacillus subtilis*	10	25	25	
R2	*Escherichia coli*	100	100	100	
M2	*Staphylococcus pyogenes* var. *aureus*	25	100	100	

*Germination was inhibited by the solvent in the control plate for 24 hours, but proceeded after this period.

Acknowledgment

The authors wish to express their gratitude to Drs. R. H. Haskins, W. A. Taber, and L. C. Vining for their helpful criticisms and to Mr. M. Granat for the infrared spectrum. The microanalyses were carried out by Mr. M. Mazurek and by Drs. Weiler and Strauss, Oxford, England. They also wish to thank Professor E. Baldacci of Milan University for a transfer of *Streptomyces caeruleus*.

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NOTE ADDED IN PROOF: Caerulomycin differs from cerulomycin, which is an antiviral antibiotic produced by *Actinomyces* (*Streptomyces*) *coerulescens*. See Chem. Abstracts, 53: 8285, 1959, for abstract of Russian paper describing cerulomycin.

GROWTH FACTOR RELATIONSHIPS OF SOIL MICROORGANISMS AS AFFECTED BY PROXIMITY TO THE PLANT ROOT[1]

F. D. Cook and A. G. Lochhead

Abstract

A comparison was made of the growth factor requirements and growth factor synthesizing capacity of bacteria from control soil, rhizosphere, and rhizoplane of wheat. Organisms for which growth factors were essential were proportionately much less abundant at or near the root than in more distant soil; this is ascribed chiefly to lower percentages of forms requiring thiamine, biotin, and vitamin B_{12}. At the rhizoplane proportions of bacteria requiring growth factors were as low as, or lower than, in the rhizosphere. Bacteria capable of synthesizing growth-promoting substances were proportionately much more numerous in the root zone than in control soil. Somewhat greater capacity was shown by rhizoplane than by rhizosphere isolates. Fungi characteristic of the rhizoplane showed the highest capacity for vitamin production.

The occurrence of growth-promoting substances at or near the root is attributable chiefly to microbial synthesis rather than to root excretion. This is the reverse of the situation believed to exist respecting amino acids. The preferential stimulation of growth factor synthesizing organisms is regarded as an indirect effect of plant growth—a reflection of the direct stimulation of amino acid requiring forms, a group with pronounced capacity for vitamin synthesis.

Introduction

There is abundant evidence that microbial growth factors are present in soil and that the soil microflora includes many microorganisms for which growth factors are essential as well as others which are able to synthesize such substances (14). Limited information is at hand on the effect of the growing plant on bacteria of both categories. Thus it has been reported (16) that the percentages of bacteria requiring vitamin B_{12} or the terregens factor were lower in the rhizospheres of three crop plants than in control soils. On the other hand organisms capable of synthesizing one or more of five growth factors were proportionately more numerous in the rhizospheres of two crops studied (13).

In the work mentioned the 'rhizosphere' referred to soil adhering to the roots. However, it is known that the highest concentrations of microorganisms normally occur on the root surface. Although these organisms doubtless contribute to the population of the rhizosphere as ordinarily sampled, they comprise the forms most intimately associated with the root itself and most under its influence. The root surface, therefore, may be considered as a special environment, distinguishable from the rhizosphere, or soil adjacent to the root. It is here referred to as the 'rhizoplane', a term first proposed by Clark (2).

The purpose of the studies here reported was to compare organisms isolated by nonselective procedures from environments of different proximity to plant roots, with respect to (*a*) their requirements for growth factors; and (*b*) their capacity for elaborating growth-promoting substances.

[1]Manuscript received March 20, 1959.
Contribution No. 482 from Microbiology Research Institute, Canada Department of Agriculture, Ottawa, Ontario.

Materials and Methods

Source of Cultures

Before analysis each group of samples was pooled. For the 'rhizosphere' samples the roots were placed in dilution blanks, following shaking to remove more loosely attached soil, and further dilutions prepared for plating. The roots were then removed, washed in three changes of sterile water, resuspended in sterile water, and ground for 3 minutes in a Waring blendor. These preparations were considered to represent samples containing organisms characteristic of the 'rhizoplane'. Appropriate dilutions were plated with soil extract agar without added energy material and the isolates (approximately 100 from each environment) obtained according to procedures previously described (16, 17).

Two experimental series were carried out:

Expt. 1. Wheat (greenhouse samples); comparison of rhizosphere and rhizoplane.

Expt. 2. Wheat (field samples); comparison of control soil, rhizosphere, and rhizoplane.

A. Growth Factor Requirements

The basal medium consisted of inorganic salts, glucose, and vitamin-free 'Casamino acids'. It was prepared by adding to 1 liter of distilled water: K_2HPO_4, 1.0 g; KNO_3, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $FeCl_3.6H_2O$, 0.01 g. The solution was adjusted to pH 6.8, heated to boiling, cooled, and filtered; 1.0 g glucose and 1.0 g 'Casamino acids' were then added.

The growth factor needs of the isolates were determined in the following stages:

(i) Each culture was first inoculated into the basal medium and into medium Y (containing salts, glucose, and yeast extract, 0.1%) and medium YS (containing, in addition, soil extract), prepared as previously described (15, 16). All strains capable of growing in the basal medium, and thus not requiring growth factors, were eliminated.

(ii) Cultures showing no growth in the basal medium or in medium Y, but growth in medium YS, included the organisms that required vitamin B_{12} or the terregens factor (TF). These were differentiated by inoculation into a series of the following media: Y (neg. control); YB_{12} (B_{12}, 0.2 μg/100 ml); YTF (TF, 10 μg/100 ml); YB_{12}TF; and YS (pos. control).

(iii) All cultures unable to grow in the basal medium were tested for specific vitamin requirements other than for B_{12} or TF. Vitamins were divided into two groups and the growth responses were noted upon the addition of each group, as well as of both groups, to the basal medium supplemented with B_{12} and TF. In this way it was possible to eliminate, in many cases, a group of vitamins as non-essential. Depending upon the results of the group tests, detailed requirements were determined by inoculation into a series of media containing, respectively, the group or groups with essential factors and the same combinations minus each vitamin in turn. Negative and positive controls were included in all tests.

The vitamins were used at the following concentrations per 100 ml of medium:

Group 1.—Thiamine, 50 μg; biotin, 0.1 μg; calcium pantothenate, 50 μg; folic acid, 10 μg; nicotinic acid, 50 μg.

Group 2.—Riboflavin, 50 μg; pyridoxine, 50 μg (with pyridoxal, 50 μg and pyridoxamine, 10 μg); *p*-aminobenzoic acid, 50 μg; choline, 2 mg; inositol, 5 mg.

The various media, dispensed in test tubes, were inoculated by loop transfer (1 mm) from suitable liquid cultures. Growth responses were normally read after 5 days' incubation at 26° C though a small proportion of more slowly growing organisms was kept longer. A growth factor was considered essential if no growth occurred on its omission from an otherwise adequate medium. Repeat tests were made in doubtful cases.

B. Synthesis of Growth Factors

Tests were made of the ability of each isolate from expt. 1 to synthesize six, and of those from expt. 2 to synthesize nine, growth factors. These consisted of thiamine, biotin, pantothenic acid, folic acid, nicotinic acid, riboflavin, pyridoxine, vitamin B_{12}, and the terregens factor. They represented the growth-promoting substances found in Part A of this study to be essential for any of the isolates.

The culture medium was similar to the basal medium described above, with the addition, per liter, of 8 mg $CoCl_2.6H_2O$ and 4.0 g, instead of 1.0 g, of 'Casamino acids'. The medium was dispensed in 40-ml amounts in 125-ml Erlenmeyer flasks. Following inoculation from 5-day cultures on soil extract yeast agar the flasks were incubated on a reciprocal shaker for 6 days at 26° C.

From all cultures showing growth, which varied from slight to abundant, test samples of the metabolic fluids were prepared. The reaction was adjusted to pH 6.4 and the flasks heated at 15 lb for 7 minutes. The cultures were then clarified by centrifugation and the clear liquids sterilized.

The general procedure for recognition of the growth factors consisted in adding to 5.0 ml of a suitable basal medium, adequate for growth of the test organism except for the factor in question, 0.5 ml of the clarified metabolic liquid, inoculating with a loopful (1 mm) of a suspension of the test organism, and incubating the tubes at 26° C (or 32° C) for 3 to 5 days. Negative and positive control tubes were inoculated in all cases. Definite growth of the test (indicator) organism in the tubes containing the filtrate was considered indicative of the presence of the growth factor in question.

The indicator organisms were all isolated from soil, either during previous investigations (1, 15, 17), or in the course of Part A of this study. None showed response to combinations of other growth factors in the absence of the factor or factors found to be essential. Essential factors other than that being tested for, as well as others which were stimulatory, were included in the respective basal media as required, with the exception of media for B_{12}

TABLE I

Indicator organisms and control media for detection of growth factors in culture filtrates

Culture No.	Growth factor requirements	Growth factor tested for	Negative control medium* (in addition to salts and glucose)	Positive control medium (addenda to negative control)
824 (*Arthrobacter* sp.)	Thiamine	Thiamine	'Casamino acids'	Thiamine (50 μg/ 100 ml)
54 (*Micrococcus* sp.)	Biotin Thiamine Vitamin B_{12}	Biotin	'Casamino acids' Thiamine Vitamin B_{12}	Biotin (0.1 μg/100 ml)
S101 (*Flavobacterium* sp.)	Pantothenic acid	Pantothenic acid	'Casamino acids' Thiamine Biotin	Ca pantothenate (50 μg/100 ml)
GR 28 (*Agrobacterium* sp.)	Folic acid Thiamine Biotin	Folic acid	'Casamino acids' Thiamine Biotin Ca pantothenate Nicotinic acid	Folic acid (10 μg/100 ml)
C 91	Nicotinic acid Thiamine	Nicotinic acid	'Casamino acids' Thiamine Biotin Ca pantothenate Folic acid	Nicotinic acid (50 μg/100 ml)
785 (*Flavobacterium* sp.)	Riboflavin	Riboflavin	'Casamino acids'	Riboflavin (50 μg/100 ml)
GR 94	Pyridoxine Thiamine	Pyridoxine	'Casamino acids' Thiamine Biotin Ca pantothenate Folic acid Nicotinic acid	Pyridoxine (50 μg/100 ml) Pyridoxal (50 μg/100 ml) Pyridoxamine (10 μg/100 ml)
541 (*Arthrobacter duodecadis*)	Vitamin B_{12} Thiamine	Vitamin B_{12}	Yeast extract	Vitamin B_{12} (0.2 μg/100 ml)
401 (*Arthrobacter flavescens*)	Terregens factor Thiamine	Terregens factor	Yeast extract	Terregens factor (10 μg/100 ml)

*Basal medium to which culture filtrates added.

and TF, to which yeast extract was added. These media served as negative controls. Positive controls included the factor under test. The indicator organisms and control media are shown in Table I.

Results

A. Growth Factor Requirements

The results of the preliminary comparison of isolates from the rhizosphere and rhizoplane (expt. 1) are shown in Table II; the requirements of bacteria isolated from control soil, rhizosphere, and rhizoplane of the field samples (expt. 2) are summarized in Table III. Whereas in expt. 1 a lower percentage of the rhizoplane isolates required growth factors than was noted for organisms from the rhizosphere, no difference was found, in expt. 2, in the proportions of isolates from these two environments that required one or more factors. The outstanding feature of the findings (Table III) was the occurrence in the control soil of a much higher proportion of organisms needing one or more growth-promoting substances than in the rhizosphere or rhizoplane.

In all cases thiamine was found to be the factor most frequently required. In addition to this vitamin, biotin and vitamin B_{12} were the most commonly needed growth substances in the control soil, the findings confirming a previous study of non-rhizosphere soil (17). In rhizosphere and rhizoplane biotin and, particularly, B_{12} were relatively much less important than in the control. Less obvious differences were noted in respect to other factors. The results suggest that the pronounced decrease in proportions of growth factor requiring bacteria at or near the root surface is ascribable chiefly to greatly diminished percentages of those for which thiamine, biotin, and vitamin B_{12} are essential.

TABLE II

Growth factors required by isolates from rhizosphere and rhizoplane (expt. 1)

	Rhizosphere (103 isolates)				Rhizoplane (100 isolates)			
	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates
Thiamine	9	12	21	20.4	10	7	17	17.0
Biotin	3	7	10	9.8	1	4	5	5.0
Pantothenic acid	3	6	9	8.7	0	1	1	1.0
Folic acid	0	3	3	2.9	0	7	7	7.0
Nicotinic acid	0	0	0	<1.0	0	1	1	1.0
Riboflavin	3	1	4	3.9	0	0	0	<1.0
Pyridoxine	0	0	0	<1.0	0	0	0	<1.0
Vitamin B_{12}	0	2	2	1.9	0	1	1	1.0
Terregens factor	0	1	1	1.0	0	0	0	<1.0
p-Aminobenzoic	0	0	0	<1.0	0	0	0	<1.0
Choline	0	0	0	<1.0	0	0	0	<1.0
Inositol	0	0	0	<1.0	0	0	0	<1.0
One or more			37	36.1			21	21.0

TABLE III

Growth factors required by isolates from control soil, rhizosphere, and rhizoplane (expt. 2)

Growth factor required	Control soil (107 isolates)				Rhizosphere (99 isolates)				Rhizoplane (100 isolates)			
	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates
Thiamine	18	30	48	44.9	8	7	15	15.2	4	13	17	17.0
Biotin	2	18	20	18.7	2	4	6	6.1	4	3	7	7.0
Pantothenic acid	0	4	4	3.7	1	2	3	3.0	1	2	3	3.0
Folic acid	0	2	2	1.8	1	2	3	3.0	0	4	4	4.0
Nicotinic acid	1	5	6	5.6	0	6	6	6.1	1	4	5	5.0
Riboflavin	0	2	2	1.8	2	0	2	2.0	2	2	4	4.0
Pyridoxine	0	2	2	1.8	0	1	1	1.0	0	5	5	5.0
Vitamin B_{12}	1	20	21	19.6	0	2	2	2.0	0	1	1	1.0
Terregens factor	0	2	2	1.8	0	0	0	<1.0	0	1	1	1.0
p-Aminobenzoic	0	0	0	<0.9	0	0	0	<1.0	0	0	0	<1.0
Choline	0	0	0	<0.9	0	0	0	<1.0	0	0	0	<1.0
Inositol	0	0	0	<0.9	0	0	0	<1.0	0	0	0	<1.0
One or more factors			58	54.2			24	24.2			25	25.0

B. Synthesis of Growth Factors

The capabilities of the isolates from the rhizosphere and rhizoplane for synthesis of six growth factors (expt. 1) are shown in Table IV. In Table V are summarized the results of the comparison of organisms from control soil, rhizosphere, and rhizoplane with respect to their ability to produce one or more of nine growth-promoting substances.

TABLE IV

Growth factors produced by isolates from rhizosphere and rhizoplane (expt. 1)

	Rhizosphere (103 isolates)				Rhizoplane (100 isolates)			
	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates
Thiamine	0	59	59	57.3	0	82	82	82.0
Biotin	0	13	13	12.6	0	25	25	25.0
Pantothenic acid	4	56	60	58.2	0	77	77	77.0
Riboflavin	4	62	66	64.1	2	83	85	85.0
Vitamin B_{12}	0	27	27	26.2	1	31	32	32.0
Terregens factor	0	16	16	15.5	0	18	18	18.0
One or more of 6 factors			72	69.9			88	88.0
1 factor			8	7.8			3	3.0
2 factors			7	6.8			8	8.0
3 factors			27	26.2			37	37.0
4 factors			18	17.5			19	19.0
5 factors			6	5.8			13	13.0
6 factors			6	5.8			8	8.0
Av. No. growth factors synthesized per active culture			3.3				3.6	

In Table V it is noted that with all factors capacity for synthesis was in all cases notably greater with bacteria from the rhizosphere and rhizoplane than with those from soil distant from the plant. Whereas 37.4% of the control isolates were able to produce one or more growth factors under the conditions of the tests, this ability was shown by 79.8% and 79.0% of those from rhizosphere and rhizoplane respectively. In both of these environments the greatest absolute percentage increases in vitamin-synthesizing bacteria were with organisms producing riboflavin and nicotinic acid. In the rhizosphere the greatest proportionate increases occurred with organisms producing pyridoxine and riboflavin, and in the rhizoplane with those forming pyridoxine and biotin. Lowest increases were recorded with bacteria forming B_{12} and TF.

Though no difference was noted in expt. 2 between the percentages of isolates from the rhizosphere and rhizoplane able to produce one or more factors, higher numbers were noted with rhizoplane isolates in expt. 1 (Table IV). With respect to the synthesis of the individual growth factors, higher

TABLE V

Growth factors produced by isolates from control soil, rhizosphere, and rhizoplane (expt. 2)

Growth factor produced	Control soil (107 isolates)				Rhizosphere (99 isolates)				Rhizoplane (100 isolates)			
	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates	Alone	With others	Total	% of isolates
Thiamine	1	29	30	28.0	0	61	61	61.6	0	68	68	68.0
Biotin	0	15	15	14.0	0	33	33	33.3	0	43	43	43.0
Pantothenic acid	0	35	35	32.7	0	71	71	71.7	2	72	74	74.0
Folic acid	1	27	28	26.2	0	58	58	58.5	0	61	61	61.0
Nicotinic acid	0	33	33	30.8	0	71	71	71.7	1	73	74	74.0
Riboflavin	0	29	29	27.1	3	69	72	72.7	0	76	76	76.0
Pyridoxine	0	20	20	18.7	0	56	56	56.6	0	58	58	58.0
Vitamin B_{12}	0	15	15	14.0	0	20	20	20.2	0	27	27	27.0
Terregens factor	0	16	16	15.0	0	23	23	23.2	0	21	21	21.0
One or more of 9 growth factors			40	37.4			79	79.8			79	79.0
1 factor			2	1.9			3	3.0			3	3.0
2 factors			2	1.9			5	5.1			2	2.0
3 factors			4	3.7			1	1.0			2	2.0
4 factors			4	3.7			4	4.0			3	3.0
5 factors			8	7.5			15	15.1			5	5.0
6 factors			4	3.7			18	18.2			16	16.0
7 factors			9	8.4			20	20.2			29	29.0
8 factors			4	3.7			8	8.1			18	18.0
9 factors			3	2.8			5	5.1			1	1.0
Av. No. growth factors synthesized per active culture		5.5				5.9				6.4		

percentages of active isolates were obtained from the rhizoplane than the rhizosphere with all six factors in expt. 1 and with eight of the nine factors in expt. 2. This finding is related to the ability of the rhizoplane organisms to synthesize a somewhat greater average number of factors than those from the rhizosphere (Tables IV, V). The results lend support to the view that as the root surface is approached the physiological activity of the microorganisms tends to increase, and are consistent with previous reports of a higher level of activity of bacteria at or near the roots than in non-rhizosphere soil (7, 8, 9, 12, 26).

Discussion

The finding of a lower percentage incidence of vitamin-requiring bacteria in the rhizosphere than in non-rhizosphere soil and equally low, or still lower, proportions at the root surface points to circumstances quite different from those prevailing in the case of amino acid requiring organisms. It has been repeatedly shown that bacteria whose demands for growth are met by amino acids are relatively more abundant in the rhizosphere than in control soil (cf. 19); moreover, it has been found that the proportions of such bacteria are further increased in the rhizoplane (11, 22). Since the effect of root excretions might be expected to be most marked at the soil–root interface, where this group is subject to the most pronounced preferential stimulation, the findings point to the plant as the dominant factor. The evidence is strengthened by the report of Katznelson *et al.* (10) that amino acids liberated from roots could be utilized by amino acid requiring bacteria, and by the finding of Gyllenberg (4) that strong development of these organisms resulted from associated growth of plants in a medium free from amino acids.

In 1939 West (24) found that detectable amounts of thiamine and biotin could be liberated by roots of flax seedlings grown under sterile conditions, a finding that favored the view, still widely held, that vitamins available to microorganisms in the rhizosphere are supplied through excretions from roots. Though this hypothesis received support in an early study from this laboratory (25) of the 'rhizosphere effect' on microbial nutritional requirements, a later investigation (20), based on the use of improved differential media and growth criteria (18), provided no evidence of a preferential stimulation of organisms for which growth factors were essential or stimulative, and so cast doubt on the significance of root excretions. The lowered proportions of growth factor requiring bacteria at or near the root surface, noted in the present study, strongly favor the view that although excretion of such factors is possible, essential growth-promoting substances are not furnished by the plant to any important degree.

Although vitamin-requiring organisms are proportionately depressed in the zone of influence of the plant, their absolute numbers are higher than in non-rhizosphere soil on account of the general 'rhizosphere effect'. Hence large numbers of such forms occur at or near the root surface. The essential factors for such organisms are believed to be supplied mainly through the

synthetic abilities of other organisms—forms which have been shown in this report to be greatly increased proportionately in rhizosphere and rhizoplane. In view of the over-all increase they may attain very high numbers.

Schmidt and Starkey (23), in studies of levels of riboflavin and pantothenic acid in soil during decomposition of added plant residues, have shown that soil microorganisms are concerned in the production of growth factors as well as in their destruction. During the early stages of decomposition of added organic matter, when the more soluble components would be preferentially attacked, the rate of vitamin synthesis was found to exceed that of destruction. It is suggested that an analogous situation, as far as being favorable to growth factor production is concerned, is provided by the liberation of amino acids from roots. These stimulate in marked fashion amino acid dependent organisms which require no external supply of growth substances but have a pronounced capacity for their synthesis.

It is considered, therefore, that although the preferential stimulation of amino acid requiring microorganisms in the region of the plant is a direct effect resulting from liberation of amino acids, that of the growth factor synthesizing forms is an indirect effect, really a reflection of the development of the former which in the present study were found to comprise most forms capable of producing growth substances. There seems no good reason to postulate a direct rhizosphere effect on microorganisms with respect to their synthetic abilities (production of growth factors, antibiotics, or other metabolic products), nor does it seem logical to do so. It is difficult to conceive of any effect other than an indirect one, through association of synthetic properties with nutritional characteristics.

It is suggested that the findings may have some relevancy to certain aspects of the nutrition of fungi—mycorrhizal or other types—associated with plant roots. Though it has been shown (cf. reviews by Harley (5, 6) and Melin (21)) that many mycorrhizal forms are dependent upon, or stimulated by, certain vitamins, divergent views are held as to the source (host or soil) from which such fungi derive any necessary nutrilites. However, in discussing vitamin deficiencies known to be prevalent among ectotrophic and ectendotrophic mycorrhizae, Melin (21) suggests that the diversity in vitamin demands indicates ". . . that in nature the main source of these metabolites for the mycorrhizal fungi may not be the host but the soil . . ." The occurrence of high numbers of growth factor synthesizing bacteria at the soil–root interface suggests that vitamins elaborated by such forms may contribute significantly to the needs not only of ectotrophic mycorrhizal fungi and other root-inhabiting forms but also of soil-inhabiting fungi in the root zone, either obligate saprophytes or unspecialized parasites (according to the ecological grouping of Garrett (3)), for some members of which requirements for vitamins have been established.

That not only bacteria but also soil fungi at or near the roots may contribute to the supply of growth factors in that region was indicated by a comparative test of the capacity of 27 species to synthesize six growth factors.

The fungi comprised groups of nine, isolated respectively from control soil, rhizosphere, and rhizoplane and regarded as representatives of the three environments. Each isolate was grown in two media under two cultural conditions, shaken and stationary, and culture filtrates obtained and tested as described above. Growth factor production was estimated on a semi-quantitative basis by comparing growth, in respect to each individual factor, with that shown by the respective positive controls. Results, based on the average 'scores' of 36 determinations, are summarized in Table VI. Though none of the fungi was able to synthesize B_{12}, and TF production, as with bacteria, was less related to the environment, the organisms from the root surface were found to be more active in producing thiamine, biotin, pantothenic acid, and riboflavin than those from other sources. The findings are therefore consistent with the belief that at or near the root surface the level of vitamin production is higher than in soil apart from the plant and that the nutrilites so formed are at the disposal of organisms requiring them.

TABLE VI

Comparative growth factor synthesizing capacity of fungi from control soil, rhizosphere, and rhizoplane (average scores, 36 determinations)

Source of fungi	Growth factor synthesis (maximum = 4)					
	Thiamine	Biotin	Panto-thenic	Ribo-flavin	Vitamin B_{12}	Terregens factor
Control soil (9 species, 9 genera)	1.3	1.4	2.6	1.9	0	2.0
Rhizosphere (9 species, 9 genera)	1.8	1.4	2.6	2.2	0	2.1
Rhizoplane (9 species, 6 genera)	2.0	2.4	3.0	2.7	0	1.8

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IDENTIFICATION OF AN ALKALINE-DEPENDENT STREPTOMYCES AS STREPTOMYCES CAERULEUS BALDACCI AND CHARACTERIZATION OF THE SPECIES UNDER CONTROLLED CONDITIONS[1]

W. A. TABER

Abstract

The identity of the *Streptomyces* which produces the new crystalline antibiotic, caerulomycin, was determined. The distinctive characteristics of the culture are: produces a blue-to-red indicator pigment; requires a neutral or alkaline reaction for growth; bears oblong to cylindrical spores in straight and flexuous chains; is not chromogenic on peptone media but produces hydrogen sulphide on iron-peptone agar; and does not grow on unbuffered potato or carrot plugs, Difco litmus milk, ASA agar, or Pridham and Gottlieb's synthetic agar medium. The culture readily utilizes glucose, fructose, galactose, mannitol, sucrose, xylose, starch, and maltose but does not utilize, or utilizes to a limited extent, mannose, i-inositol, adonitol, lactose, ribose, raffinose, and cellulose.

A direct comparison with authentic cultures of *Streptomyces coelicolor* and *Streptomyces caeruleus* proved the unknown to be qualitatively indistinguishable only from the latter. A literature survey did not reveal other species resembling the unknown, which was therefore concluded to be a member of *Streptomyces caeruleus* Baldacci.

The original description of *S. caeruleus* is amended and some characteristics of the culture grown under reproducible conditions are reported.

Introduction

A *Streptomyces* (PRL 1687) which produced a new crystalline antibiotic, caerulomycin (5), was recently isolated in this laboratory. As a result of a direct comparison with living cultures in the laboratory the culture was identified as *Streptomyces caeruleus* Baldacci.

Although *S. caeruleus* was described by Baldacci in 1944 (i.e. *Actinomyces caeruleus*) (2), the species has not been listed, either as a valid species or a synonym, in Bergey's Manual (4) or Waksman and Lechevalier's monograph (14). Krassilnikov (7) describes the species in his monograph but the description evidently is his own since it does not agree with the original description of Baldacci. Baldacci found that the culture requires a pH of at least 8 for growth while Krassilnikov states that it grows on conventional media. The latter also reports that the blue pigment does not diffuse through the agar, but diffusion was observed in the investigation reported in this paper.

Thus, one confronted with the problem of identifying an isolate of *S. caeruleus* would not be able to do so unless in possession either of an authentic isolate or of the original description appearing in the Italian journal (2). Further, since it was found through examination of an authentic isolate of *S. caeruleus* obtained from Baldacci that the culture would grow at a pH

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lower than 8 (but not lower than 7), identification might not be made even if the original description were available. In addition, defined media were not employed in the original description and it therefore would not be possible to reproduce the original conditions.

The identification of PRL 1687 as *S. caeruleus* and comparison of it with a well-known reference culture, *Streptomyces coelicolor*, is reported, firstly to demonstrate that the species is distinct from others and therefore should be recognized as a species; secondly to report some characteristics of the culture when grown under reproducible conditions; and thirdly to report that while a neutral or alkaline pH is required, the limiting pH is less than the reported 8.

Materials and Methods

Media

2A.—Since the culture would not grow on Pridham and Gottlieb's medium, another synthetic medium, 2A (10), was employed for the determination of the carbohydrate utilization pattern. The basal medium, which contained asparagine and salts, was adjusted to pH 7.2 before autoclaving. The carbon sources were autoclaved separately. The brands of carbohydrate are listed below in conformity with a suggestion put forth at the "Round Table Conference on Streptomyces" (8): Difco galactose (purified as described previously (11)), Fisher reagent anhydrous dextrose, Difco levulose, Baker and Adams reagent mannitol, NBC adonitol, BDH certified *l*-inositol, NBC USP beta lactose, Fisher reagent sucrose, Fisher reagent D-mannose, NBC xylose, Difco raffinose, Matheson, Coleman, and Bell maltose, NBC ribose, Mallinkrodt soluble starch, and Whatman powdered cellulose. The media were dispensed in 50-ml quantities in 250 ml Erlenmeyer flasks. The medium was later modified (2A-pH 8) by decreasing the concentration of dibasic potassium phosphate to 3.0 g/liter, omitting the monobasic phosphate, and adjusting the pH to 8.2. When a solid medium was required, the completed, sterile medium was mixed with dry, sterile agar and melted on a boiling water bath.

Gelatin.—An autoclaved solution of Difco gelatin was mixed with sterile 2A and 2A-pH 8 sugar-free media to give a final concentration of 6% gelatin.

WGA.—Waksman glucose broth (13) supplemented with agar to 1.5% was adjusted to pH 7.1 before autoclaving. This medium was previously (10) referred to as No. 3 agar.

DTA, ASA, GAA, synthetic medium.—These were prepared as described by Anderson *et al.* (1).

Difco iron peptone agar.—The agar medium was supplemented with yeast extract according to Tresner and Danga (12).

Carrot and potato plugs.—Cylinders were cut with the cork borer and autoclaved in test tubes. After cooling, sufficient sterile water was added to cover the lower quarter of the cylinder. Buffered plugs were prepared by replacing distilled water with 2 ml of 20% K_2HPO_4.

Inoculum

All media were inoculated with mycelia obtained from shaken cultures grown in glucose-2A-pH 8 (or 2A) medium. These cultures were started with spores from soil cultures. Liquid media were inoculated with 0.2 ml, and agar media with the quantity of suspension adhering to a bacterial loop.

Growth Conditions

Flasks of inoculated liquid media were incubated at 30° C on a Gump shaker revolving at 112 r.p.m. Agar cultures were grown in incubator rooms held at 30° C.

Estimation of Growth

Growth was measured as dry weight per flask culture (10) and is reported as the arithmetic mean and standard error of three replicate cultures. The approximate 95% confidence interval can be obtained by multiplying each standard error by 4.3 ($t_{.05}$ at 2 degrees freedom).

Assay of Caerulomycin

The presence of activity against the test organism, *Candida albicans* ATCC 10231, was detected by dipping paper disks in culture filtrates and applying to plates of WGA agar seeded with the test organism. Three spot checks[2] showed the activity to be due to caerulomycin and it was therefore assumed that this was the source of antifungal activity of all active cultures.

Results

The presence of a diffusing blue-to-red indicator pigment and blue aerial mycelia suggested that the unknown was a member of *Streptomyces coelicolor* and an authentic isolate was obtained[3] for a direct comparison in the laboratory. The cultural characteristics of PRL 1687 and *S. coelicolor* differed markedly (Table I). The spores of PRL 1687 were cylindrical or oblong and the chains were flexuous or straight while the spores of *S. coelicolor* were near globose and were borne as coils.

Although the above data were sufficient to demonstrate that PRL 1687 was not *S. coelicolor*, their carbohydrate utilization patterns were compared in order to determine whether they could also be distinguished on this basis (Table II). Their patterns clearly differed. The pattern of *S. coelicolor* agreed with that reported by Benedict *et al.* (3). The resemblance of PRL 1687 to *S. coelicolor*, then, is confined to pigmentation, and this resemblance is superficial since the pigments are not identical.

A comparison of PRL 1687 with other species was made through literature comparisons (3, 4, 7, and 14). Since universally accepted species criteria do not exist (see 6, 8, and 15), morphology, pigmentation, and nutritional characteristics were selected as a matter of personal preference. Using these criteria,

[2]Performed by Dr. P. V. Divekar, Postdoctorate Fellow, Prairie Regional Laboratory, Saskatoon, Sask.

[3]*S. coelicolor* NRRL B-1257 was kindly supplied by Dr. T. G. Pridham, Principal Mycologist, Agricultural Research Service, Peoria, Illinois.

TABLE I

Comparison of *Streptomyces* PRL 1687 with two known blue species

Medium	PRL 1687	Authentic *Streptomyces caeruleus*	Authentic *Streptomyces coelicolor*
Waksman's glucose broth agar (WGA)	Abundant growth. Spore mass purplish purple blue (7.5 PB 4/2).* Reverse purple-blue, purple-blue diffusing pigment. Spore chain flexuous. Spore (dry) oblong; 1.5–2.4× 0.8 μ	Abundant growth. Spore mass purplish purple blue (7.5 PB 5/2). Reverse purple-blue, purple-blue diffusing pigment. Spore chain flexuous. Spore (dry) oblong; 1.5–2.4× 0.8 μ	Abundant growth. Spore mass blue (7.5 B 4/4). Reverse blue. Blue diffusing pigment. Spore (dry) globose to oval. 0.6–0.8 μ
DTA	Abundant growth. Culture indistinguishable from above. Spore chain flexuous but short	Abundant growth except for light patches of spores, indistinguishable from PRL 1687 on DTA agar. Spore chain flexuous but short	Abundant growth. Spore mass blue (7.5 C 4/4). Dark blue exudate. Reverse purple, purple diffusing pigment. Spore chain open spiral
GAA	Growth limited. Spore mass pale lavender. Reverse green-blue, diffusing pigment green-blue. Spore chain flexuous	Growth limited. Spore mass lavender-gray. Reverse gray. No diffusing pigment. Spore chain flexuous	Abundant growth. Spores and aerial hyphae brick red. Reverse dark red. Red diffusing pigment. Spore chain open spiral
ASA	No growth	No growth	Abundant growth. Spore mass blue (7.5 B 4/4). Reverse blue, blue diffusing pigment. Spore chain open spirals and loops
Pridham & Gottlieb's synthetic	No growth	No growth	Abundant growth. Spore mass and aerial hyphae pale green. Reverse brown. No diffusing pigment. Spore chain open spiral
Difco litmus milk	No growth	No growth	Abundant growth. Blue pigment. Alkaline hydrolysis without coagulation
Difco iron peptone agar + yeast extract	Abundant growth. Positive sulphide test. Spore mass gray, later blue	Abundant growth. Positive sulphide test. Spore mass gray, later blue	Abundant growth. Negative sulphide test. Colorless vegetative mat

Starch agar (2A-pH 8)	Hydrolysis positive. Center of spore mass lavender, margin white. Blue diffusing pigment	Hydrolysis positive. Same appearance as PRL 1687	Hydrolysis positive, but weak. Spore mass blue. Reverse blue and diffusing blue pigment
Glucose 2A-pH 8	Abundant growth. Spore mass at first white, then becoming blue. Reverse blue and blue diffusing pigment	Identical with PRL 1687	Vegetative mat, reverse and diffusing pigment purple. Spore mass cream becoming pale blue
Potato plug	No growth†	No growth	Abundant growth. Vegetative mat red with some blue aerial patches
Carrot plug	No growth‡	No growth	Abundant growth. Vegetative mat colorless and spore mass white
Gelatin 2A-pH 8	Restricted growth. Non-chromogenic	Restricted growth. Non-chromogenic	Restricted growth. Non-chromogenic

*Munsell book of color.
†Growth occurs on potato plugs buffered with a solution of K_2HPO_4 to pH 8.2.
‡Slight growth when buffered.

TABLE II

Comparative carbohydrate utilization of PRL 1687 and *Streptomyces coelicolor* (basal medium of 2A)

Carbon source	PRL 1687*						*Streptomyces coelicolor**					
	4 days		7 days		11 days		4 days		7 days		11 days	
	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH
Glucose	16.9±1.4	7.1	72.4±5.1	7.0	109.5± 4.6	6.9	182.0±41.3	6.5	198.5 ± 29	6.7	258.6 ±27.3	6.5
Fructose	19.3±2.6	7.1	54.6±3.4	7.0	91.9± 5.1	6.9	117.5± 2.1	7.4	248.7 ± 6.3	6.6	288 (single value)	6.1
Galactose	14.4±0.4	7.2	54.9±6.1	7.0	60.8± 5.1	6.9	104.5±10.1	7.8	154.2 ± 7.1	7.8	370.2 ±83.2	7.1
Mannitol	No growth†	7.1	No growth	7.0	25.9± 5.5	7.1	62.2± .78	7.1	248.4 ± 3.7	6.6	221.14± 9.8	6.8
i-Inositol	No growth	7.3	No growth	7.1	No growth	7.2	No growth	7.2	11.1 ± 0.7	7.1	34.7 ± 5.1	7.2
Sucrose	10.4±2.7	7.0	22.5±5.3	7.3	125.1±23.6	7.5	71.2± 3.6	7.4	68.5 ± 2.1	8.4	63.7 ±10.8	8.9
Mannose	No growth	7.0	No growth	7.0	No growth	7.0	138.4±10.0	6.9	342.7 ±23.6	6.4	276.1 ±12.6	6.8
Maltose	7.8±1.1	7.0	21.5±4.5	7.2	214.3± 5.7	6.8	191.7± 8.1	6.8	203.7 ± 4.8	7.2	201.3 ±10.0	7.3
Xylose	No growth		12.9± .54	7.0	45.1± 2.43	6.6	14.1± 1.2	7.0	269.44±38.4	6.7	286.54±27.4	7.2
Ribose	No growth		No growth	6.5	No growth	6.0	208.3± 2.9	5.5	194.36±22.8	6.4	72.06± 6.2	7.3
Raffinose	No growth		No growth	7.2	45.1± .9	7.4	No growth	7.2	31.36±31.8	7.4	64.55± 7.3	8.1
Adonitol	No growth		No growth		No growth	7.3	No growth		No growth		No growth	7.3
Lactose	No growth		No growth	7.3	No growth	6.9	13.6± 3.58	7.1	113.7 ±11.9	7.0	249.0 ± 5.0	6.1
Starch	36.5±1.7	7.1	133.0±0.6	7.0	53.1± 6.1	7.3	48.6± 1.38	5.8	222.8 ± 6.3	7.3	169.2 ±10.1	7.9
Cellulose	No growth		No growth		No growth		No growth		No growth		No growth	

*Caerulomycin was produced by PRL 1687 on all media allowing abundant growth. *S. coelicolor* did not produce any activity toward *Candida albicans*.
†Since cultures grown on the basal medium to which no carbon source was added attained approximately 10 mg growth, growth on an added carbon source is concluded to have occurred only when the value exceeds this number.

PRL 1687 was concluded not to be *S. coelicolor*, *S. cyaneus*, *S. violaceus*, or *S. pluricolor* because these species bear spores in coiled chains. It is not *S. caelestis* because the latter bears spores in hooks and grows readily on conventional media. PRL 1687 is not a member of *S. vinaceus* because it possesses spherical spores, and is not a member of *S. viridis* (Lombardo-Pellegrino) because the latter grows on conventional media and produces a green pigment. A close resemblance does appear to exist, however, since both *S. viridis* and PRL 1687 possess cylindrical spores and PRL 1687 does produce a green-blue pigment under certain circumstances (see footnote of Table IV). PRL 1687 might be identical with the pH dependent, straight-chained strains of *S. coelicolor* referred to by Krassilnikov (7), but details of these are not available so further comparisons cannot be made.

The characteristics of PRL 1687 placed it in the section Rectus-Flexibilis, series blue of the scheme of Pridham *et al.* (9). It seemed possible that PRL 1687 could be *Streptomyces caeruleus* Baldacci, and when it was learned[4] that *S. caeruleus* requires an alkaline pH for growth, an authentic isolate was obtained from Professor E. Baldacci, Milano, Italy, for a direct comparison in the laboratory.

The cultural characteristics of PRL 1687, authentic *S. caeruleus*, and *S. coelicolor* were determined simultaneously using the same batches of media (Table I). *S. coelicolor* was included to serve as controls for the cultural conditions. Except for the intensity of pigmentation, PRL 1687 and *S. caeruleus* could not be separated, while again *S. coelicolor* was clearly different from PRL 1687. The carbohydrate utilization patterns of PRL 1687 and *S. caeruleus* were determined using basal medium 2A-pH 8 and they were qualitatively indistinguishable (Table III). The ability of PRL 1687 and *S. caeruleus* to utilize mannitol but not mannose under the conditions employed seemed unusual but the same results were obtained upon repetition of the test. The possibility that the bottle of mannose had become contaminated with toxic material was ruled out when it was found that *S. coelicolor* grew readily on media containing this source of mannose.

Authentic *S. caeruleus* also produced an antibiotic (Table III) and it was isolated and compared with caerulomycin produced by PRL 1687.[5] The antibiotic of *S. caeruleus* melted at 175° C, did not depress the melting point of caerulomycin isolated from PRL 1687, and had infrared and ultraviolet absorption spectra identical with those of caerulomycin.

It was concluded that PRL 1687 was an isolate of *S. caeruleus* Baldacci. For convenience, PRL 1687 was referred to as the Canadian isolate, and authentic *S. caeruleus* as the Italian isolate.

Quantitative Difference Between the Canadian and Italian Isolates

If the two isolates were identical, then it should not be possible to separate them into two groups on any basis. However, when cultures grown on WGA

[4]Dr. O. Ciferri, Postdoctorate Fellow, Prairie Regional Laboratory, Saskatoon, Sask.
[5]Isolation and characterization performed by Dr. P. V. Divekar, Postdoctorate Fellow, Prairie Regional Laboratory, Saskatoon, Sask.

TABLE III

Comparative carbohydrate utilization by PRL 1687 and *Streptomyces caeruleus* (basal medium of 2A-pH 8)

Carbon source	PRL 1687*				*Streptomyces caeruleus**			
	3 days		11 days		3 days		11 days	
	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH	Mg dry weight	pH
Glucose	319.6±15.2	7.6	191.6±13.9	7.7	380.1±26.3	8.1	229.8±15.2	7.5
Fructose	231.8±11.0	7.7	201.6± 8.6	7.0	197.1±25.2	8.0	220.0±17.1	7.5
Galactose	182.1±30.2	8.0	232.5±15.3	7.7	339.1± 5.0	8.1	202.2±16.3	7.9
Mannitol	226.8±23.2	7.9	266.1±11.1	7.9	200.6±16.4	7.8	199.2± 7.6	7.8
Adonitol	No growth		No growth		No growth		No growth	
Inositol	No growth		No growth		No growth		No growth	
Lactose	No growth		No growth		No growth		No growth	
Sucrose	132.6± 3.7	8.0	206.5± 0.4	7.5	122.5± 1.8	8.2	261.5±11.5	7.7
Mannose	No growth	7.8	Trace	8.0	No growth	7.8	Trace	8.0
Xylose	185.3±10.6	7.2	262.5± 0.5	7.2	81.4±18.1	7.2	248.5±23.5	7.2
Raffinose	29.8± 0.5†	8.5	23.2± 3	9.0	Trace†	8.6	Trace	8.8

*Both cultures produced caerulomycin on glucose, fructose, galactose, mannitol, sucrose, and xylose.
†The limited growth may be due to utilization of a contaminating carbon source or to use of only a portion of raffinose.

agar were coded and randomized, the mixture could be divided into two groups, on the basis of pigment intensity, and these groups corresponded to the two isolates. It was concluded, however, that this quantitative difference, which might have been due to the fact that one strain had been under cultivation in the laboratory several years and the other but 1 year, does not warrant erection of subspecific names.

The Alkaline pH Requirement

Baldacci (2) reported that *S. caeruleus* would not grow when the pH of the medium was below 8.0. Buffered potato and carrot plugs allowed growth. PRL 1687 also grew on buffered potato and carrot (Table I) but not on buffered Pridham and Gottlieb's medium, possibly because the concentration of phosphate required to raise the pH was toxic. Under the conditions employed in this study, both isolates would grow in media adjusted to pH 7.2 but not 6.9 (Tables III and IV), and thus the "alkaline requirement" reported by Baldacci was confirmed but the lower limit of pH 8.0 was not.

TABLE IV

Effect of initial pH on growth (basal medium 2A-pH 8; 6 days)

Initial pH	Mg growth		Final pH	
	Canadian isolate	Italian isolate	Canadian isolate	Italian isolate
6.9	None*	None*	6.8	7.1
7.6	96.7±8.9	68.4±17.1	7.8	7.9
8.0	91.9±2.8	82.2± 7.1	8.2	8.3
8.2	125.6±8.1	86.2±10.8	8.1	8.4
8.6	71.0±8.8	74.5± 1.5	8.4	8.6
9.6	56.6±3.5	49.6± 1.0	8.8	8.9

NOTE: The cultures started at pH 9.6 were pink, all others were green-blue.
*No growth by 20 days.

Acknowledgment

Grateful acknowledgment is extended to Miss Charlotte Marshall for technical assistance, to Dr. T. G. Pridham, Principal Mycologist, Agricultural Research Service, Peoria, Illinois, for providing an isolate of *Streptomyces coelicolor* NRRL B-1257, and to Professor E. Baldacci, Director, Laboratory of Plant Pathology, Milano, Italy, for providing an isolate of *Streptomyces caeruleus*.

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NOTE ADDED IN PROOF: Caerulomycin differs from cerulomycin, which is an antiviral antibiotic produced by *Actinomyces* (*Streptomyces*) *coerulescens*. See Chemical Abstracts 53: 8285, 1959, for translation of the Russian paper describing cerulomycin.

STUDIES ON HAEMOBARTONELLA INFECTION IN THE RAT[1]

ARTHUR C. FORD, JR.[2] AND T. J. MURRAY

Abstract

An organism which answers the published descriptions of *Haemobartonella muris* was isolated from Sprague-Dawley rats. The rats had either been splenectomized or were under other forms of stress. The organism was grown in a serum–tryptone medium which had been used successfully in the cultivation of *Bartonella bacilliformis*. The cultivated organism was infective for non-carrier Sprague-Dawley rats under different forms of stress; Wistar rats were not carriers and infections could not be induced.

The effects of protein intake and tumors on the development of infection are also discussed. Rats fed a diet of 12% casein while carrying Flexner-Jobling tumors were more susceptible to infection than were those on diets of 0, 6, or 18% casein. Neither diets alone nor tumors alone placed sufficient stress on the animals for the development of symptoms.

Many attempts have been made to cultivate the etiologic agent of murine haemobartonellosis. There is, however, no uniformity in various reports, which are summarized in the excellent review by Weinman (11), who is of the opinion that none of the reports of cultivation are completely satisfactory. The present paper deals with results obtained during a recent study of *Haemobartonella* infection in the rat. The culture medium in which initial isolates were obtained was that used by Gieman (5) in the cultivation of *Bartonella bacilliformis*.

The characteristics of *Haemobartonella* infection have been described by many investigators (11). The chief manifestation is a severe anemia characterized by the appearance of hematuria and hemoglobinuria. Splenomegaly is another characteristic. Death usually occurs in about 10 days following the removal of the spleens of latently infected rats.

Previous work with tumors seemed to indicate that mortality following splenectomy was less in cancerous rats than in non-tumor-bearing rats (4, 7, 8). In our work it was demonstrated that Sprague-Dawley rats implanted with the Flexner-Jobling tumor were, in general, more susceptible to *Haemobartonella* infection.

Little work has been reported on the effects of diet on the development of *Bartonella* infection in the rat. Wills and Mehta (12) reported that combined A and C avitaminosis in the non-splenectomized rat provoked a severe *Bartonella* anemia. Hadju (6) found that diets deficient in vitamin D, when fed to rats 1 to 4 weeks before splenectomy, doubled the subsequent mortality. McCluskie and Niven (9), using an unspecified but insufficient diet, did not obtain more haemobartonellosis than with a complete diet.

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Contribution from the Department of Bacteriology and the Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey, U.S.A. Part of a thesis submitted in partial fulfillment for the degree of Doctor of Philosophy.
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Materials and Methods

Liquid Serum–Tryptone Medium

This medium was composed of 75 ml of a 1% aqueous solution of tryptone, pH 7.6–7.8, 25 ml sterile, fresh, rabbit serum, and 0.2 ml of sterile (Seitz-filtered) ascorbic acid – glutathione solution. The medium was prepared in the manner recommended by Gieman (5) and dispensed to sterile 50-ml flasks in 10-ml amounts.

Two-tenths and 1.8% agar was added to make semisolid and solid media respectively.

Cultivation Procedure

Heart blood was drawn from infected rats and 0.5 ml was added to 10 ml of culture medium in 50-ml Erlenmeyer flasks. The cultures were incubated at 28° C and examined daily for evidence of growth.

Serological Methods

Conventional agglutination tests were employed with the bacterial antigens. The precipitin test was used with the rickettsial antigen. The antigen was an extract of *Rickettsi prowazekii* and was supplied by Lederle Laboratories, Pearl River, New York.

Antibiotic Sensitivity Test

The agar plate-disk method was used in these studies.

Animals

(*a*) The Sprague-Dawley (Madison, Wisconsin) strain of albino rats was used in all of the experiments reported here.

(*b*) The infection was induced in the experimental animals by intraperitoneal injection of blood from experimentally infected rats, also by splenectomy of carrier rats.

(*c*) Flexner-Jobling tumor material was obtained from carrier rats, minced, and introduced subcutaneously by the trochar method.

(*d*) Conventional haematological techniques were used to determine the extent of the anemia.

Diets

The casein diets were those employed by Allison *et al.* (1). Semisynthetic diets were prepared containing 0, 6, 12, and 18% casein respectively. The composition of these diets is shown in the report referred to above. The diets and water were offered ad libitum.

Experimental Groups

Groups consisting of six animals on experiment and six controls were set up. The experimental groups were housed in separate cages.

Results and Discussion

Cultivation

An organism which answers the published descriptions of *Haemobartonella muris* was isolated from 11 of 20 splenectomized Sprague-Dawley carrier rats and from 16 of 24 carriers bearing Flexner-Jobling tumors.

The control groups gave negative results in each case.

It was found that 72 hours following the inoculation of Gieman's medium with the blood of the various rats, a thin film of growth occurred at the bottom of the flasks. Giemsa stains and wet mounts revealed small coccoid bodies. This film of growth was rather tenacious and was spread on the slide with some difficulty.

Small portions of the film were transferred to fresh medium and again incubated at 28° C. At the end of 72 hours, the bottoms and walls of these flasks were coated with growing organisms giving a ground-glass appearance. A few small clumps appeared free in the broth. The growth from the bottom and sides of the flasks was suspended with difficulty, even when the flasks were placed on a shaking machine.

Material from these flasks was streaked to slants of solid serum–tryptone medium. Some of the growth was also streaked on solid medium in Petri dishes. The tubes and plates were incubated at 28° C, and in 72 hours small colonies appeared on the surface of the solid medium. These increased in size on further incubation, reaching a maximum size of from 0.5 to 1 mm in diameter. The surface of the colonies presented a rough wax-like appearance, resembling somewhat the colonies of acid-fast organisms. Upon subsequent transfers, the colonies became smooth in texture. When single colonies were picked for stains and subcultures, they revealed the same morphological type of organism, namely, a small diplococcus. The organism was infective for splenectomized Sprague-Dawley rats; for rats carrying Flexner-Jobling tumors and on 12% casein diets; and for rats receiving injections of 4 mg of cortisone[3] per kilogram of body weight per day.

The Organism

The organisms appeared to be cocci, occurring singly or in pairs. They ranged in size from 0.2 to 4.2 μ. The larger forms were found in old cultures and appeared to be clumps of more than one cell. Individual cells measured from 0.2 to 0.6 μ in diameter.

The organisms gave a negative reaction when stained by Gram's method, and they stained purple or pinkish-purple with Giemsa stain.

They were non-motile, non-acid-fast, non-filterable, and appeared to be non-encapsulated. Optimum growth was obtained at a temperature of 28° C. Some growth occurred at 37° C, but the lower temperature appeared to be the optimum. Best growth was obtained in an alkaline environment. The pH range for growth was determined to be from 7.0 to 7.8, with optimum conditions being afforded in the range of from 7.4 to 7.6. The organism is aerobic, growing best in shallow layers of broth, and at the surface of semisolid media. No growth was obtained under anaerobic conditions. It is somewhat fastidious in growth requirements. No growth occurred in the absence of enrichments. It was also found that certain sera were better than others in growth-promoting properties. Human, bovine, and horse sera were substituted for the rabbit

[3]Product of Merck-Sharpe & Dohme.

serum in Gieman's medium in one series of experiments. It was found that no growth occurred in the human serum, and relatively little growth was obtained with the bovine and horse sera. The rabbit serum gave the best growth.

Serological studies showed that the organism was weakly antigenic in the rabbit. An agglutinin titer of 1:1024 was obtained by a double immunizing series of injections. This antiserum agglutinated *Proteus* OX-19 and *Proteus* OX-K, giving titers of 1:64 in each case. No reaction was obtained in a cross-agglutination test with *Streptobacillus moniliformis*. (Dr. John B. Nelson of the Rockefeller Institute of Medical Research had suggested that the organism might have been an "L" form of *Streptobacillus moniliformis*.) Negative results were also obtained when a precipitin test employing a soluble *Rickettsia prowazekii* antigen was performed.

As indicated previously, this organism, isolated from anemic rats, shows many of the characteristics which have been described for *Haemobartonella muris*.

A recent review by Peters and Wigand (10) shows the salient characteristics of the organism of murine haemobartonellosis. Table I shows how the organism which we have been able to cultivate compares with the descriptions of the above authors.

TABLE I

Comparison of the characteristics of the organism from anemic rats with that given by Peters and Wigand (10)

	Haemobartonella muris	Organism from anemic rats
Giemsa stain	Red-purple	Pinkish-purple
Morphology	Coccoid	Coccoid and diplococcoid
Motility	None	None
Size	0.3 to 0.5 μ	0.2 to 0.6 μ
Filterability	None	None
Serology:		
Antiserum reaction with rickettsial antigen	None	None
With *Proteus* OX-19 and *Proteus* OX-K	Occasionally positive, titers of doubtful significance	Same
Drug sensitivity:		
Sulfa compounds	None	None
Penicillin	No	Yes
Streptomycin	No	No
Chloramphenicol	No	Yes
Aureomycin	Yes	Yes
Terramycin	Yes	No

The discrepancies in drug susceptibility may be due to the fact that previous studies dealt with chemotherapy in vivo, and not with in vitro tests as performed here.

On the basis of the evidence presented here, it is possible that the organism which we have been able to cultivate is *Haemobartonella muris*. We have injected the organism into rats under stress and have recovered the same or a

similar organism in artificial culture. Weinman (11) indicated that reports of the cultivation of *H. muris* should rule out the possibility of the survival of viable organisms from the original inoculum.

We suggest that, since the organism here was not reisolated from rats in the absence of some sort of stress, that question has been answered.

Effect of Diet and Tumors

As noted earlier, little work has been reported on the effect of diets and tumors on the development of haemobartonellosis in the rat. We were unable to find any reports dealing with the effects of various levels of protein in the diet on the development of the anemia. In the present work, a series of experiments employing casein diets were performed. Young Sprague-Dawley rats weighing 100 to 125 g were used. They were injected with blood from infected rats on the day before being placed on the diets. The diets were fed ad lib. Groups consisting of six experimental animals and six controls were set up in each experiment. It was found that diet alone had little or no effect on the course of the infection. If, however, previously infected Sprague-Dawley rats were inoculated with Flexner-Jobling tumors and placed on a diet of 12% casein, 90 to 100% of the experimental animals developed a severe hematuria in about 10 days. The blood cell changes in these rats were characteristic of haemobartonellosis. Cultures from those rats showing symptoms were positive. The same results were obtained in repeated experiments.

If, on the other hand, 6% casein, 18% casein, or a protein-free diet were employed, either alone or in conjunction with the tumor, the animals did not develop hematuria. Cultures were negative.

The results obtained in these experiments are in accord with those obtained in another series of experiments. Allison *et al.* (2) and Allison (3) have shown that the development of the Flexner-Jobling carcinoma in the rat is greatly affected by the protein in the diet. It was found that optimum development of the tumor occurred in rats fed a 12% casein diet. The induction period was shortened, and maximum size was reached sooner on this diet than on any other containing less or more casein.

This suggests that this level of dietary casein in the presence of the tumor produces effects of the same order of magnitude as produced by splenectomy.

In all of the experiments reported, it was necessary to rule out the possibility of latent infections in the control groups. This has been done by reporting only those results where the controls were consistently negative. It was found that with some litters of Sprague-Dawley rats, no reliable results could be obtained. This was due to the presence of latent infections.

When Wistar rats were employed in parallel experiments, it was found that no infection could be induced, and that cultures from such rats were always negative. The tumor employed in the Wistar rat experiments was the R-I sarcoma of the Rutgers Bureau of Biological Research. The Wistar rat is not susceptible to the Flexner-Jobling tumor.

The results of these experiments suggest the possibility that certain tumors in higher animals may serve to aggravate latent infections—if a sufficient amount of stress accompanies the tumor.

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MOTILE STREPTOCOCCI ISOLATED FROM THE OROPHARYNGEAL REGION[1]

RUDOLPH HUGH

Abstract

Five strains of motile, alpha hemolytic streptococci were isolated during a systematic study of 820 oropharyngeal specimens for group D streptococci. The motile streptococci contain the Lancefield group D somatic polysaccharide. Photomicrographs record the presence of flagella in stained preparations. It appears both practical and expedient to classify these motile group D streptococci as another variety of *Streptococcus faecalis*.

Introduction

The genus *Streptococcus* is sometimes erroneously considered to be exclusively composed of nonmotile and nonflagellated strains. Flagellated streptococci are thought of as being rare and confined to a few saprophytic forms. These concepts may have evolved simply from failure to examine streptococci for the presence of flagella and motility. Several hundred motile streptococcus strains have been described (1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 16, 22, 23, 24, 25, 27, 28, 29). Motile streptococci have been isolated from water, feces, urine, blood stream, inflamed heart valves, and are of bovine, mouse, and human origin.

Materials and Methods

This report concerns the description of six strains of motile streptococci. Five motile strains[2] were encountered among 70 group D streptococci isolated during a systematic study of 820 oropharyngeal tonsil regions (15). The method of isolation and study was described by Hugh *et al.* (15). The sixth strain was the motile *Streptococcus faecalis* ATCC 12755 studied by Sherman (28).

Results

Strains 12817 and 12819 were isolated from a patient undergoing treatment for pernicious anemia. The oropharyngeal flora of this subject also contained a coliform, with + − + + IMViC reaction, belonging to the Oxytoca group (14). Strain 12818 was isolated from a patient with untreated advanced ulcerated epidermoid cancer of the gingival ridge, and this specimen also contained *Escherichia coli*. Two strains were isolated from a patient after surgical treatment of *in situ* cancer of the soft palate. One of these strains was encountered 8 months after surgery while the other was isolated 11 months after surgical treatment, at which time there was no evidence of

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[2]Three strains have been deposited with the American Type Culture Collection and designated 12817, 12818, 12819.

disease except for leucoplakia. *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* (21) were also isolated from the oropharyngeal region of this patient. It is interesting that the motile group D streptococci were isolated from three of the 523 patients with disease. They were not found in the group of 297 normal individuals studied.

The microbes grew abundantly in brain heart infusion broth with an even smooth turbidity and produced a slight sediment. Growth on nutrient agar was very scant. Rabbit blood agar deep colonies were surrounded by a zone of green alpha hemolysis. The size and consistency of surface blood agar colonies gave the general appearance of nonmotile nonpigmented group D streptococci.

The organisms appeared as Gram-positive cocci in pairs, often elongated and lanceolate in shape when stained from 18-hour cultures in brain heart infusion broth. Long chains of cocci form in bile broth. Flagella were readily stained by the technique described by Leifson (19).

The photomicrographs, Figs. 1–8, illustrate the flagellation of all the strains. Generally only one or two flagella were seen on a single coccus. Cocci occasionally appeared with three and, more rarely, five flagella. Flagella generally appeared to originate at the distal ends of the lancet-shaped pairs; however, it was not uncommon to see flagella extending from the point of junction of the cocci in pairs. The length of the flagella varied from 1 to 10 times the diameter of the coccus. The wave length and amplitude of the flagella appeared to be similar to that seen in *Salmonella wichita* (19). Short flagella with single curves were often seen (Figs. 2, 3, and 7). The shapes of these short flagella resemble the flagella of *Lophomonas alcaligenes* (20 and 7).

Vigorous motility was best seen in early logarithmic phase cultures at room temperature. Strain 12755 was nonmotile at 37° C. These observations were confirmed by observing the extent of diffuse growth in semisolid agar.

The results of the biochemical and serological tests of these microbes are recorded in Table I. None of the strains fermented adonitol, dulcitol, or inositol. Dextrose was fermented without gas production. These organisms failed to exhibit gelatinolytic activity in both nutrient and charcoal gelatin

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FIG. 1. Motile *Streptococcus faecalis* showing six cocci in a chain with two flagella. Photomicrograph ×2000.

FIG. 2. Motile *Streptococcus faecalis*, a single coccus with two flagella, one short and one long. Photomicrograph ×2000.

FIG. 3. Motile *Streptococcus faecalis* showing a pair of lanceolate cocci with two flagella, one long terminal and one short lateral flagellum originating from a single coccus. Photomicrograph ×2000.

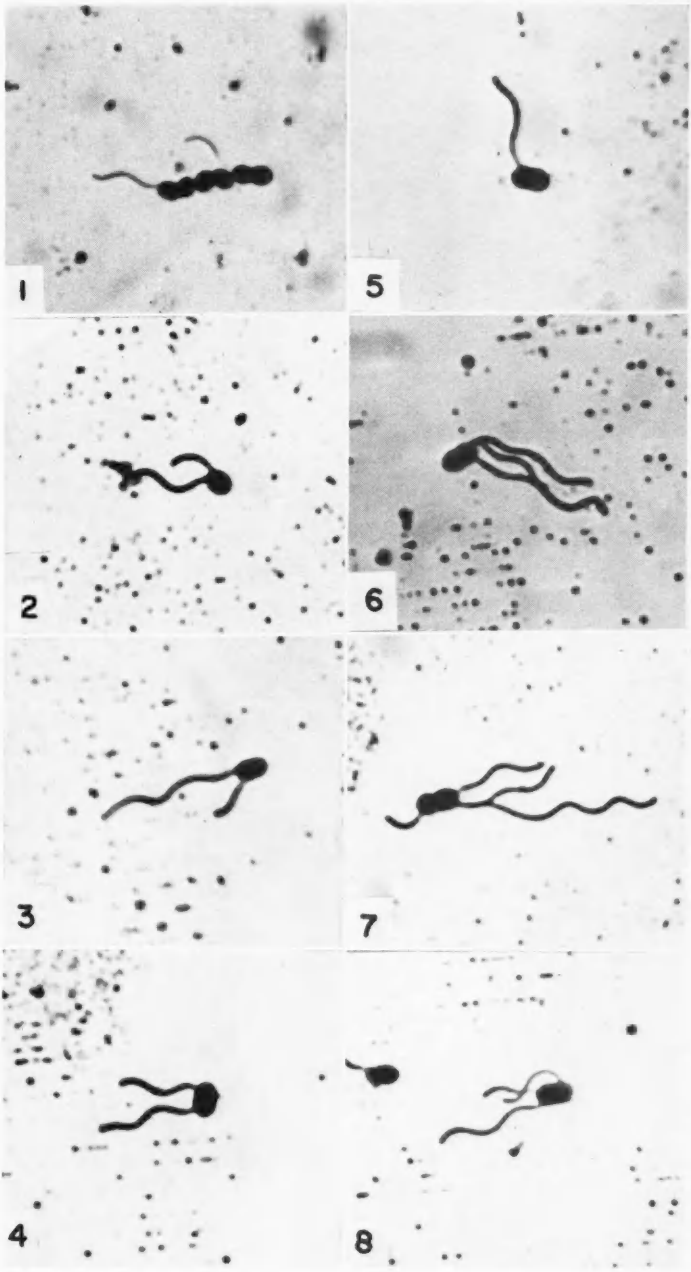
FIG. 4. A pair of motile lanceolate *Streptococcus faecalis* with a lateral flagellum originating from each coccus. Photomicrograph ×2000.

FIG. 5. A pair of motile lanceolate *Streptococcus faecalis* with a single lateral flagellum. Photomicrograph ×2000.

FIG. 6. Motile *Streptococcus faecalis* showing a pair of lanceolate cocci with three long flagella, two terminal and one lateral flagellum originating from a single coccus. Photomicrograph ×2000.

FIG. 7. Motile *Streptococcus faecalis* showing a pair of lanceolate cocci with four flagella, three of which are long and the fourth short. Photomicrograph ×2000.

FIG. 8. A pair of lanceolate *Streptococcus faecalis* with three long flagella. Photomicrograph ×2000.



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(18) media. Catalase was not produced. The capillary precipitin test carried out with an autoclaved extract of the cells (26) gave a more rapid and a stronger reaction than when performed with a hot hydrochloric acid extract (17) of the cells.

TABLE I

Cultural behavior of six motile group D streptococcus strains

Arabinose	6+*
Dextrose	6+
Lactose	6+*
Maltose	6+
Mannitol	6+
Rhamnose	3+*, 3−
Salicin	6+
Sorbitol	1+, 6−
Sucrose	6+*
Trehalose	6+*
Xylose	6+
Inulin	4+*, 2−
Raffinose	3+*, 3−
Aesculin	6+*
6.5% Sodium chloride broth growth	6+
Ethyl violet azide broth growth	6+
Motility	6+
Resistant to 60° C for 15 minutes	6+
Resistant to 60° C for 30 minutes	4+, 2−
Rabbit blood agar deep colonies—alpha hemolysis	6+
0.1% Methylene blue milk reduction	5+, 1−
0.1% Methylene blue milk coagulation	5+, 1−
40% Bile aesculin agar growth	6+
40% Bile aesculin agar—color black	5+, 1−
Precipitin test—Lancefield HCl extract	6+

NOTE: − indicates a negative reaction,
+ indicates a positive reaction,
* reaction often delayed for more than 1 day.

Discussion

Motility and flagellation should be looked for when studying streptococci. Leifson's method of staining bacterial flagella in our hands was both adequate and convenient, although Auerback and Felsenfeld (1) were unsuccessful in applying the method to visualize the flagella of motile streptococci.

The described motile group D streptococci are like nonmotile *Streptococcus faecalis* in that both produce negative adonitol, dextrose gas, dulcitol, inositol, and catalase test reactions; with positive dextrose, lactose, maltose, salicin, trehalose, aesculin, 6.5% sodium chloride broth, ethyl violet azide broth, 40% bile-aesculin agar growth, and precipitin test reactions (15). Motile strains of *Streptococcus durans* were not encountered. It appears that all motile streptococci contain the Lancefield group D somatic polysaccharide. A thermolabile H (flagellar) antigen was demonstrated by Graudal (9, 10) which agglutinated and immobilized most of the strains he studied.

The reactions listed in Table I are interpreted to indicate that a marked physiological difference does not exist between the motile and nonmotile group D streptococci, and this difference is not sufficient to warrant a distinctive species epithet for the motile strains. The author considers the motile group D streptococci as merely another variety of *Streptococcus faecalis*.

Nonmotile *Streptococcus faecalis* and motile group D streptococci have been differentiated (10, 11, 12) on the basis of yellow pigment, a variety of physiological reactions, and also by type-specific somatic antigens. These differences seem to represent the extremes of variation within the concept of the species rather than a species difference.

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INFLUENCE OF THIAMIN ON GROWTH AND FAT FORMATION BY PENICILLIUM LILACINUM THOM.[1]

KAISER NAGUIB

Abstract

Penicillium lilacinum was grown on a high sugar–salts medium favorable for fat formation. Thiamin hydrochloride was added to 5-day-old cultures at three different concentrations, namely, 0.25, 2.5, and 5.0 mg/l. Sugar uptake, growth, and fat formation were followed after addition of vitamin for a period of 9 days. Sugar uptake was enhanced by the lowest and suppressed by the two higher vitamin concentrations. At higher concentrations, there was an early inhibition of dry weight increase, but by the end of the experiment, the weight of the high thiamin cultures equalled that of the controls. The effect of thiamin on fat formation was dependent on both the concentration and period of incubation. By the end of the incubation period, the low thiamin concentration had promoted fat formation, the high concentration had suppressed it, while the medium concentration was ineffective.

Introduction

The effect of vitamins on fat formation has long been investigated in relation to animal feeding (Whipple, Church, and Stevens (26), McHenry and Gavin (11, 12), etc.). With microorganisms, several workers have obtained higher yields of cellular material through addition of vitamins to cultural media, e.g. Odintsova (16), Olson and Johnson (17), and White and Munns (27). In an attempt to promote fat formation in molds through addition of vitamins, Woodbine (30) reported higher fat yields by adding thiamin alone in cultures of *Penicillium javanicum* and *Penicillium soppi*. Using a mixture of vitamins, he got a rather suppressive effect. Naguib (13), using riboflavin and nicotinic acid in cultures of *Penicillium lilacinum*, obtained higher fat contents with riboflavin but not with nicotinic acid.

The present work represents an attempt to find out whether it is possible to promote fat synthesis by *Penicillium lilacinum* through the addition of thiamin. It was also thought that the present study may throw some light on the effect of thiamin, when added at different concentrations, on the growth of this mold; the work on this subject by various authors and with different microorganisms led to different results depending on the various experimental conditions.

Materials and Methods

The Organism

The fungus used in the present work, *Penicillium lilacinum* Thom., was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Cultural Conditions and Composition of Media

For keeping stock cultures and for inoculation purposes, the fungus was grown on Dox's agar slopes and subcultured every 2 weeks. In the experimental work, surface cultures were grown in 100-ml conical flasks each containing

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Contribution from the Botany Department, Faculty of Science, University of Cairo, Egypt.

25 ml of a basal medium of the following composition (g/100 ml): sucrose, 17; sodium nitrate, 0.64; sodium dihydrogen phosphate, 0.73; potassium sulphate, 0.011; magnesium sulphate, 0.5; zinc sulphate, 0.005; and ferric chloride, 0.016. The pH of the medium was adjusted with sodium hydroxide, before distribution into the culture flasks, to 6.8 This medium was found by Naguib and Walker (14) to promote fat formation by *Penicillium lilacinum*. The flasks were plugged and sterilized at 15 lb for 10 minutes. They were then inoculated with a spore suspension prepared from 2-week-old slant cultures, and incubated at 25° C. After 5 days, when complete surface mats have just been developed, the vitamin as thiamin hydrochloride solution was added cautiously and aseptically beneath the felts. It was found advisable to add the vitamin when active fat synthesis started to take place. This was found, by preliminary experiments, to be after 5 days from inoculation. Three concentrations of the vitamin were used, namely, 0.25, 2.5, and 5.0 mg/l. The culture flasks were incubated again. Estimation of the sugar content of the culture media, the dry weight, as well as the fat content of the mycelial felts were carried out directly on addition of the vitamin solution and then after 3, 6, and 9 days. At these intervals, five replicates were withdrawn for analysis.

Methods of Analysis

The mycelial felts were recovered by filtration, dried at room temperature for 2 days and then under vacuum at 70° C for an hour, and then weighed.

Estimation of sugar in the filtrate was carried out by a modified Schaffer-Hartmann's method in the manner described by Saïd and Naguib (22).

Estimation of fat in the felt was carried out as given by Woodbine, Gregory, and Walker (31).

Results

Sugar Uptake

The rate of sugar uptake, as can be noted from the drop in sugar concentration in the culture media (Fig. 1), was accelerated at the lowest concentration of thiamin used, but was suppressed at the higher concentrations. In this connection, it may be mentioned that Dammann and co-workers (1) using *Gibberella saubinettii* and Hawker (6) using *Melanospora destruens* found that these fungi could use glucose at a higher rate when thiamin was added to the culture medium.

Dry Weight of the Mycelial Felts

It can also be noted from Fig. 1 that the dry weight of the mycelial felts increased at a higher rate in the presence of the lowest concentration of thiamin than in the thiamin-free cultures. In the two higher concentrations, the rate of growth was first suppressed, the suppression being greater with the higher concentration, but by the end of the experiment, the dry weights of these mycelial felts were quite near to those of the vitamin-free cultures. Odintsova (16) has reported promotion of growth by thiamin. Schopfer and Guilloud (25), on the other hand, have reported an inhibitory effect of thiamin on

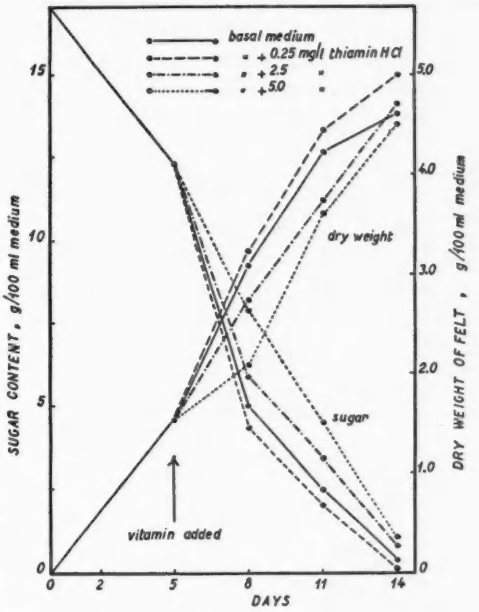

FIG. 1. The sugar content of the culture media and the dry weights of the mycelial felts during the incubation period.

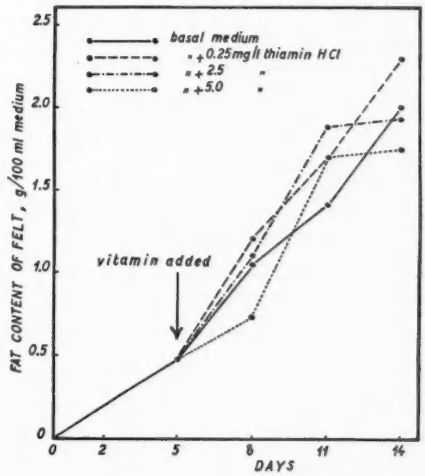

FIG. 2. The fat content of the mycelial felts during the incubation period.

growth of *Rhizopus suinus*. It is interesting to mention that these workers found that thiamin caused a stimulatory effect at first, followed by an inhibitory stage but later the rate of growth reached that of the vitamin-free cultures.

Fat Contents of the Mycelial Felts

From Fig. 2, it can be seen that thiamin, in the first 3 days, accelerated fat formation at the two lower concentrations only; the highest concentration was rather suppressive. Later, fat content was higher in all thiamin-fed cultures than in the controls, but by the end of the experiment the lowest concentration of thiamin gave the highest fat yield, the highest concentration gave the lowest yield, while the medium concentration was very near to the control.

When the fat content was calculated as percentage of the dry weight of felt (Table I), it was found that thiamin at 2.5 mg/l gave a felt containing about 50% fat in the 11-day-old cultures, compared with 34% in the control samples.

TABLE I

Effect of addition of thiamin hydrochloride to cultures of *Penicillium lilacinum* on percentage fat in felt

Vitamin concentration, mg/l	Days of incubation			
	5*	8	11	14
	Percentage fat in felt			
0	30.40	33.38	34.30	44.20
0.25		36.03	38.27	46.43
2.50		40.70	50.00	41.17
5.00		36.36	46.90	38.97

*The vitamin was added after 5 days of incubation.

Discussion

Addition of thiamin to the culture media of *Penicillium lilacinum* accelerated fat formation at the lowest and suppressed it at the two higher concentrations used. Woodbine (30) obtained an appreciable increase in fat on addition of thiamin to cultures of *Penicillium javanicum* and *Penicillium soppi* but not with *Aspergillus nidulans*. It may be of interest to refer to the work of McHenry and Gavin on young rats (11) and on pigeons (12). They succeeded in promoting fat synthesis by these animals when thiamin was added to their diet.

However, a large amount of work has been carried out on the effect of thiamin on the growth of microorganisms, but it has led to different conclusions. Thus, using thiamin-deficient organisms, acceleration of growth has been recorded by several workers, e. g. Schopfer (23) with *Phycomyces Blakesleeanus*, Knight (8) with *Staphylococcus aureus*, Kögl and Fries (9) with *Nematospora Gossypii*, Robbins (20) with *Phytophthora* spp., Robbins and Kavanagh (21)

with *Pythium Butleri*, Noecker (15) with wood-destroying fungi, and Fothergill and Ashcroft (4) with *Venturia inaequalis*. When added to self-sufficient organisms, thiamin in some cases manifested a growth-promoting effect. Odintsova (16) found that the growth rate of *Torula utilis* was doubled by addition of thiamin to the culture media. In other cases, it produced an inhibitory effect as with *Fusarium lini* (Wirth and Nord (28)), *Rhizopus cohnii* (Schopfer and Guilloud (25)), and *Colletotrichum lindemuthianum* (Mathur and co-workers (10)).

Several workers such as Haag and Dalphin (5), and Wirth and Nord (29), have shown that deficiency of thiamin leads to accumulation of pyruvic acid. When thiamin is added to the culture media, pyruvic acid gradually disappears. This was explained on the assumption that thiamin, being a constituent of the coenzyme of the carboxylase enzyme system, increases the carboxylase activity, and so pyruvic acid, resulting from the breakdown of the sugar or the carbon source provided in the culture medium, is decarboxylated at a higher rate when an external supply of thiamin is added. This was further confirmed by the fact that a higher rate of respiration was detected in the presence of thiamin (Hills (7), Schopfer (24), Hawker (6), and others).

In the present investigation, however, the effect of thiamin on the metabolism of *Penicillium lilacinum* was a function of concentration. At low concentrations, a higher rate of enzymatic activity was produced. This was manifested in a higher rate of sugar absorption and subsequent consumption in growth and respiration. It may be assumed that when pyruvic acid is decarboxylated at a higher rate to yield acetaldehyde, in the presence of an adequate supply of thiamin (0.25 mg/l), the acetaldehyde produced subsequently starts, in fat-forming molds, the chain of reactions leading to fat synthesis. Hence, there was a higher rate of fat accumulation at this vitamin concentration. Fat formation via C_2 compounds (as acetaldehyde) has been postulated and confirmed by many workers (cf. Foster (2)).

At higher concentrations, on the other hand, thiamin caused an early and appreciable drop in growth rate. This may be partly ascribed to the inhibition by thiamin at these concentrations, of sugar absorption and partly by assuming that in the presence of the higher concentrations of thiamin there was an increase in the respiration rate at the expense of absorbed sugar, due to a higher carboxylase activity. It is probable that acetaldehyde formed in the later period of incubation was not directed towards fat synthesis but was reduced to ethanol which accumulated in the external media and was later used up as a carbon source in the building up of cellular material, mostly other than fat. This eventually resulted in the very slow rate of fat synthesis in this period in the presence of the high vitamin concentration. This may also explain why the dry weight of the felts fed with the high thiamin concentration could catch up to the controls by the end of the experiment. Schopfer and Guilloud (25) working with *Rhizopus suinus* discovered higher amounts of alcohol in thiamin-inhibited cultures than in the controls. Foster (3) mentioned a similar situation with *Rhizopus nigricans*. It may also be mentioned in this connection that Ramachandran and Walker (19) obtained higher yields

of alcohol in the presence of thiamin by *Candida Krusei*. The use of alcohol accumulated in old cultures has been early reported by Pritham and Anderson (18). They found that *Fusarium lycopersici* later uses up ethyl alcohol produced during metabolism on glucose, and that it is able to use the alcohol better for mycelium building than as source of energy.

Acknowledgment

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UTILIZATION OF 1-AMINO-2-PROPANOL BY A SOIL BACTERIUM[1]

S. F. GOTTLIEB[2] AND M. MANDEL

Abstract

An aerobic, motile, Gram-negative bacterium of uncertain taxonomy has been isolated from soil by the enrichment method. This bacterium utilizes DL-1-amino-2-propanol as its sole source of carbon, nitrogen, and energy for growth in mineral media. The oxidation of the aminopropanol by resting cells is complete in the presence of 2,4-dinitrophenol. Lactic acid and a number of two and three carbon substrates are oxidized with molecular oxygen by resting cells and serve as adequate substrates for the growth of the bacterium, in the presence of ammonium chloride. The amino acids alanine and aspartic acid were found to be suitable growth substrates while serine, 3-amino-1-propanol, isopropylamine, and mono- and di-methylaminoethanol did not suffice. The results of this investigation are discussed with reference to a possible cycle of vitamin B_{12} synthesis and degradation in the soil.

Introduction

The D-1-amino-2-propanol moiety of vitamin B_{12} has not been observed elsewhere in nature and the mechanism of its degradation is unknown. It has recently been shown (4) that L-threonine is the source of this portion of the vitamin during its synthesis.

A cycle of vitamin B_{12} synthesis, utilization, and degradation occurring in the soil may be inferred from the abundance of vitamin B_{12}-requiring bacteria found in the soil and the presence of both the vitamin and bacteria capable of synthesis thereof (5, 6).

This paper describes the nutrition and respiratory behavior of a soil bacterium which utilizes DL-1-amino-2-propanol as a sole source of carbon, nitrogen, and energy. The original intention, before the structure of vitamin B_{12} was known, was to obtain recognizable fragments of vitamin B_{12} by cleavage of the molecule at the aminopropanol bridge (2). Our interest resides in the ecology and metabolic reactions of the bacteria attacking aminopropanol.

Experimental Methods

Isolation Procedures

One-gram aliquots of soil were inoculated into 25 ml of sterile enrichment medium (Table I) contained in 125-ml cotton-plugged Erlenmeyer flasks. Incubation was at 22–25° C. Forty-eight hours after growth became evident, a loopful of material was aseptically transferred into a second flask which also contained the sterile enrichment medium. Forty-eight hours after turbidity appeared in the second enrichment flask, a loopful of material was streaked on enrichment agar (Table I). Single colonies were picked and restreaked on

[1]Manuscript received April 3, 1959.
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TABLE I

Composition of the different synthetic culture media used in these studies

	Medium*		
	Enrichment, %	Stock culture, %	Basal, %
KH_2PO_4	0.04	0.04	—
K_2HPO_4	—	—	0.02
Nitrilotriacetic acid	0.01	0.02	0.02
NH_4Cl	—	—	0.05
$MgSO_4.7H_2O$	0.05	0.06	0.06
Trace metal solution‡	0.1 ml	0.1 ml	0.1 ml
Ca (as $CaCl_2$)	1.5 mg	1.5 mg	1.5 mg
Mo (as $Na_2MoO_4.7H_2O$)	0.01 mg	0.01 mg	0.01 mg
Fe (as $FeSO_4.7H_2O$)	—	0.01 mg	0.01 mg
V (as $Na_3VO_4.16H_2O$)	—	0.002 mg	0.002 mg
Ethylphosphoric acid†	—	0.5 ml	0.5 ml
Thiamine HCl	—	0.05 mg	—
DL-Glutamic acid	—	0.1	—
DL-1-Amino-2-propanol	0.4 ml	0.25 ml	—
Final pH (with KOH or H_2SO_4)	6.6–6.8	6.9–7.2	6.9–7.1

*For semisolid medium add 0.3% agar, for solid medium add 1.6% agar.
†Commercial ethylphosphoric consisted of 55% diethylphosphoric acid and 45% monoethylphosphoric acid.
‡Trace metal solution contained the following per ml: 2.5 mg ethylenediaminetetraacetic acid, 2.5 mg Zn^{++}, 1.0 mg Fe^{++}, 0.5 mg Mn^{++}, 0.1 mg Cu^{++}, 0.05 mg Co^{++}, as the sulphates; and 0.02 mg B as H_3BO_3.

enrichment agar after 48 hours' incubation. Single, well-isolated colonies from the second streaking were picked and transferred to enrichment agar slants. Purity of the culture was checked by restreaking on enrichment agar and on blood agar, and by microscopic examination of gram-stained smears.

Stock cultures were maintained on chemically defined solid and semisolid media (Table I). Subcultures were made every 6 months.

Nutritional Requirements

The culture techniques employed for the study of nutritional requirements of the organism have been previously described (1). Five hundredths milliliter of a 24-hour stock culture, incubated at 34° C in semisolid medium, was diluted 1000-fold in appropriately deficient, sterile basal medium (Table I). Experimental flasks were inoculated with one drop of the dilute suspension and were incubated for 4 to 6 days at 34–36° C. Growth was measured photometrically with a Welch Densichron photometer containing a red-sensitive photocell and recorded in optical density units.

Respirometric Studies

Oxygen uptake and CO_2 production were measured by the conventional Warburg direct method. In all cases the total volume of each vessel was 3.4 ml. Vessels were incubated in a constant-temperature water bath set at 34° C and were shaken at a rate of 77 cycles per minute.

Preparation of Cell Suspensions

Ten-milliliter amounts of basal medium (Table I) supplemented with the appropriate substrate were dispensed into 125×16 mm screw-capped tubes.

After sterilization and inoculation the tubes were incubated for 24 hours at 37° C. The contents of the tubes were then transferred into 500-ml Erlenmeyer flasks containing 250 ml of sterile medium. After 24 hours' incubation at 37° C the cells were harvested by centrifugation and were washed twice with 50 ml of 0.85% NaCl. The cells were then resuspended in 50 ml saline and refrigerated. Immediately before using, the cells were again concentrated and resuspended in *M*/30 phosphate buffer at pH 6.9.

Results

Thirteen aminopropanol utilizers were isolated. One strain (P6) was chosen for detailed study because of its vigorous growth on the isolation medium and routine peptone media. On blood agar at 32° C 48-hour-old colonies were gray, dull, umbonate, with irregular lacerate edges and a gyrose structure. Similar cultures incubated at 37° C yielded cream-colored colonies with concentric rings, a pulvinate elevation with lobate edges and a cloudy structure. At both temperatures the periphery of the colonies were often lighter colored than the centers. In 48 hours the diameters ranged between 0.4 and 0.6 cm. Microscopic examination did not reveal differences between cells taken from the center and the periphery of colonies grown at the two temperatures. In tryptone water (1.0% w/v) and on blood agar the cells were small Gram-negative rods occurring singly, in pairs, and in small chains. In thioglycolate broth (casein digest plus yeast extract plus glucose, plus 0.05% sodium thioglycolate) the cells were mainly in long chains. The organism was intensely motile but all efforts to discern the flagellar distribution were unsuccessful; control preparations with cultures of the genera *Serratia* and *Proteus* were successful. P6 is intensely aerobic but produces no catalase. A heavy pellicle is formed on sugar broths. An alkaline reaction is formed with the monosaccharides arabinose, fructose, galactose, rhamnose, and xylose; with the polysaccharides dextrin and raffinose; with the disaccharides lactose, maltose, sucrose, and trehalose; with the alcohols adonitol, dulcitol, inositol, mannitol, and sorbitol; and with the glucoside salicin. In glucose broth a slightly acidic reaction is first observed, rapidly becoming alkaline. Nitrate is not reduced to nitrite, urea is not hydrolyzed, indol and acetylmethylcarbinol are not produced, and gelatin is not liquefied.

Because of our inability to obtain a satisfactory flagella stain, we cannot classify the organism with assurance. According to Skerman's key for the generic identification of bacteria (7), strain P6 keys out to be a member of the genus *Achromobacter*.

In the respirometric studies on the oxidation of 1-amino-2-propanol, 73% of the theoretical oxygen uptake was obtained. In the presence of 2,4-dinitrophenol there was an oxygen uptake corresponding to complete oxidation while there was no increase in the endogenous oxygen uptake in the presence of the drug. In the presence of 2,4-dinitrophenol the respiratory quotient was 0.86; the number of moles of oxygen consumed per mole of amino propanol oxidized was 3.7; the number of moles of carbon dioxide produced per mole of

amino propanol oxidized was 3.2. The incomplete oxidation presumably reflected assimilation and not a partial oxidation of the molecule although the higher than theoretical respiratory quotient would tend to argue against this view. Attempts to isolate the lower fatty acids and aldehydes from the reaction mixture were unsuccessful. Throughout these studies the racemic mixture of 1-amino-2-propanol was used.

The oxidation of aminopropanol does not appear to be induced by the substrate. Cells grown on yeast autolyzate, propylene glycol, acetate, or succinate

TABLE II

Nitrogen sources for the growth of P6 in basal medium

Addition		Growth (O.D.) in the presence of		
		None	Na acetate.$3H_2O$, 0.25%	Propylene glycol, 0.25%
None		0.00	0.04	0.02
NH_4Cl	0.01%	0.02	0.22	0.32
	0.05%	0.02	0.32	1.22
	0.1%	0.02	0.32	2.00
KNO_3	0.01%	0.02	0.16	0.24
	0.05%	0.02	0.22	0.88
	0.1%	0.02	0.22	1.50
1-amino-2-propanol	0.05%	0.40	—	—
	0.1%	0.70	—	—
	0.25%	1.58	—	—

TABLE III

Utilization of substrates as carbon and energy sources for the growth of P6 in basal medium*

Addition†	Growth (O.D.)
None	0.07
1-Amino-2-propanol	1.08
3-Amino-1-propanol	0.07
iso-Propylamine	0.07
Glycerol	0.14
DL-Lactic acid	0.96
Na_2 succinate	0.50
DL-Serine	0.08
DL-Alanine	0.96
DL-Aspartic acid	0.44
Na acetate	0.42
Sarcosine	0.12
Betaine	0.74
K_3 citrate	0.28
Dimethylaminoethanol	0.08
Methylaminoethanol	0.08
Propylene glycol	1.22
K glycerophosphate	0.04

*Supplemented with NH_4Cl 0.05%.
†Final concentration of each substrate: 0.25%.

oxidized aminopropanol with no lag. Washed cell suspensions stored at 5° C for 1 week lost the ability to oxidize aminopropanol. Activity was restored upon the addition of calcium D-pantothenate. Further aging again resulted in a loss of oxidative ability which could be restored by a mixture of the known water-soluble vitamins.

Bacterium P6 and 11 of the remaining aminopropanol-oxidizing bacteria are prototrophic and able to derive all their carbon, nitrogen, and energy from the dissimilation of aminopropanol. One culture was found to be auxotrophic for thiamine.

Ammonium and nitrate ions serve as adequate nitrogen sources for the growth of P6 when suitable carbon and energy sources are available as can be seen from Table II.

Table III presents a summary of the ability of the compounds tested to replace aminopropanol as carbon and energy sources for the growth of P6.

Discussion

The respirometric results indicate complete oxidation of DL-aminopropanol and raise the question: is the attack on the "unnatural" L-isomer begun by its conversion to the D-isomer? The absence of adaptive behavior in this organism—cells grown in the absence of aminopropanol oxidize DL-aminopropanol promptly—makes it impractical to apply the simultaneous adaptation technique to illuminate this and other possible stages in dissimilation.

At the time these experiments were done, pure vitamin B_{12} was too expensive for tests of its breakdown by the bacterium. Free DL-1-amino-2-propanol may be an artifact in much the same sense that free riboflavin is an artifact when compared with the natural riboflavin phosphates. Even should the organism prove able to break down vitamin B_{12}, it would be necessary to show that one point of attack was indeed at the aminopropanol bridge; present microbiological and chemical assay methods do not permit real proof of such an attack. The complexity of the vitamin B_{12} molecule is such that its complete degradation seems beyond the capacity of any one microorganism. One would expect, rather, that the vitamin B_{12} molecule would be attacked piece-meal by a conjunction of organisms whose most obvious activities would include degradation of ribose and porphyrins; degradation of the benziminazole moiety might be carried out by organisms with abilities to attack purines or riboflavin. An attack on an ultra-micronutrient such as vitamin B_{12} might be viewed, then, as the result of peripheral activities of a host of organisms, not one of them specialized to destroy vitamin B_{12} as such. To test this, one would have to examine the dissimilatory abilities of vitamin B_{12}-destroying organisms isolated with vitamin B_{12} enrichments.

By this line of reasoning, the "true" (i.e. quantitatively most important) natural substrates of bacterium P6 are unknown. In one direction, the utilization of DL-propylene glycol (but not glycerol) hints at a specialization for glycol substrates. This idea is weakened by the utilization of creatine and betaine (but not ethanolamine and mono- and di-methylethanolamine, glycine,

serine, and sarcosine). Den Dooren de Jong (3) had found choline and sarcosine to be used by only a few of his soil isolates. The inertness of sugars and 3-amino-1-propanol is another evidence of specialization.

Lochhead and Burton (5) noted that 19.2% of their isolates of "indigenous" soil bacteria needed thiamine. It is not surprising that one of the 13 aminopropanol oxidizers in the present study needed thiamine.

The ease of isolation of aminopropanol oxidizers would make it a simple matter to see whether the nutritional patterns just sketched applied to most aminopropanol oxidizers. There is no reason to suppose that aminopropanol oxidizers are a homogeneous group or even belong to new species.

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THE EFFECTS OF D-AMINO ACIDS ON ALCALIGENES FECALIS[1]

CYNTHIA LARK AND K. G. LARK

Abstract

D-Amino acids have been found to induce protoplast-like forms (crescents) in *Alcaligenes fecalis* strain LB. This induction is similar in most respects to that by penicillin with the exception of the effects produced in tryptone. D-Amino acids appear to act synergistically with penicillin. The amino acid composition of cell walls of D-amino acid and penicillin-induced crescents as well as of normal cells has been examined by column chromatography. The main difference between normal and crescent cell walls consists of a reduction in the amount of the basic peptide unit of the phenol-insoluble fraction. These results are discussed in relation to the induction of protoplast-like forms.

Introduction

Cell walls of some bacteria contain D-amino acids which are either derived directly from the medium or formed by racemization of their L-homologues. D-Amino acids are not required for growth of such cells (5, 18).

Most biological reactions involving amino acids are specific for the L-isomer only and appear to be indifferent to the D-isomer. A few inhibitory effects of D-amino acids have been reported (3, 12, 17). Recently we have observed the formation of protoplast-like forms (crescents) of *Alcaligenes fecalis* strain LB in media containing D-amino acids. Such forms are easily induced by penicillin (8), an antibiotic which contains D-dimethyl cysteine as part of its chemical composition (2). The effects of D-amino acids on *Alcaligenes fecalis*, LB, are described in this paper.

Materials and Methods

Bacterial Strain

The strain studied was *Alcaligenes fecalis* strain LB (8).

Growth Media

The tryptone or defined casamino acid media used in these experiments have been described previously (8). The L-amino acid medium is that used by Medill and O'Kane (10) with L-amino acids substituted for DL at one-half the concentrations of the latter. Methionine and aspartic acids have been omitted from this medium.

Measurements of Culture Density

Methods used in making viable colony counts and in microscopic counting as well as in measuring protein, ribonucleic acid (RNA), and desoxyribonucleic

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acid concentration have been described (8, 6). Turbidimetric measurements were made at 595 mμ in a Bausch & Lomb Spectronic 20 Spectrophotometer.

Cell Wall Analyses

Cultures for cell wall analysis were centrifuged at room temperature and the cells in the pellet lysed by resuspension in 10 ml of 1% Dupanol (1). Cell walls prepared by Dupanol lysis of bacteria appear in the phase contrast microscope as faint shadowy outlines of the bacillary form. Chemical studies have shown that Dupanol treatment removes a large amount of contaminating material (apparently protein) from the cell wall itself (7). After 30 minutes' incubation at 37° C, the suspension was diluted up to 40 ml with water and centrifuged at 5000×*g* for 10 minutes to remove unlysed cells and then at 25,000×*g* for 60 minutes. The pellet obtained was again resuspended in H_2O, homogenized in a Waring blendor, and recentrifuged at 25,000×*g*. After two additional such washings the pellet was lyophilized. The powder thus obtained, which contained no RNA, was suspended in 10–20 ml of 90% phenol (15) and incubated at 37°–45° C for 24 hours. The suspension was then centrifuged at 8000–9000 r.p.m. and the supernatant (phenol-soluble fraction) reserved. The precipitate (phenol-insoluble fraction) was resuspended in 10 ml of 90% phenol and left for 12 hours at 37°–45° C. Usually two extractions were sufficient to remove all phenol-soluble protein from the cell wall preparations. After centrifuging, the supernatants were combined and dialyzed against water until the phenol was removed. A tan precipitate remained which was washed several times with alcohol and finally with ether.

The phenol-insoluble portion was washed 2–3 times with alcohol and once with ether. As much carbohydrate as possible was removed by extracting three times with a boiling saturated aqueous solution of picric acid (4). An insoluble protein picrate remained which was washed twice with acetone – 1% HCl to remove picric acid and finally with ether. A gray powder remained after drying.

Protein hydrolyzates were prepared by weighing portions of protein into glass ampules, adding 2 ml of glass-distilled 20% HCl, and sealing the ampule. They were heated in an oven for 18 to 20 hours at 110° and then evaporated to dryness in a vacuum desiccator. The hydrolyzates were dissolved in 0.6 ml of water; .4 ml was put on 150 cm columns and .2 ml on 15 cm columns of Amberlite IRC 50. Preparation of chromatographic columns and subsequent chromatographic analysis was carried out as described by Moore *et al.* (11).

Results

Ability of Different D-*Amino Acids to Induce Crescent Formation*

Aliquots of an exponentially growing culture of *A. fecalis* were diluted in growth medium containing doubling dilutions of various D-amino acids. Growth was allowed to take place with aeration at 37° C for 2 to 3 hours, and the cultures were then checked microscopically for the presence of crescents.

In Table I various D-amino acids are listed which have been tested for their ability to induce crescent formation. In all cases where crescent formation

TABLE I

Effects of various D-amino acids on strain LB of *Alcaligenes fecalis* together with the minimum concentration of the D-amino acid necessary to produce the observed effect

D-Amino acid	Effect	Threshold concentration, *M*
Valine	Crescent induction (C.I.)	0.004
Methionine	C.I.	0.004
Phenylalanine	C.I.	0.008
Leucine	C.I.	0.008
Threonine	C.I.	0.008
Isoleucine	C.I.	0.008
Serine	C.I.	0.015
Histidine	C.I.	0.015
Tryptophane	C.I.	0.015
Alanine	C.I.	0.015
Lysine*	C.I.	0.05
Arginine	C.I.	0.06
α-Amino butyric*	C.I.	0.008
Aspartic†	No effect	Up to 0.06
Glutamic†	No effect	Up to 0.06
Proline*	No effect	Up to 0.15

*DL-Form of amino acid was used. Threshold concentration here refers to one-half DL concentration necessary to produce an effect.
†In a previous preliminary report, D-glutamic and D-aspartic acids were thought to cause bacteriostasis. This was an effect of lowered pH and not specific.

occurred, concentrations of D-amino acid above a certain threshold value were found to be totally effective, whereas those below this threshold had almost no effect. These threshold concentrations are listed in the table. Of the D-amino acids tested, only proline, aspartic, and glutamic acids did not induce crescent formation.

When comparable or even larger amounts (up to .03 *M*) of L-amino acid were added to the dilutions of the corresponding D-isomer, the threshold concentration in Table I remained unchanged. The same threshold values were also obtained when all L-amino acids were omitted from the medium and replaced by ammonia as a nitrogen source.

Because of the high potency of D-methionine as a crescent inducer, it was used in further studies on crescent induction.

DNA, RNA, and Protein Synthesis in Cells Being Transformed into Crescents by D-*Methionine*

D-Methionine was added to an exponentially growing culture (generation time = 40 minutes) at 0 minutes. The culture was then incubated with aeration at 37° C and samples were withdrawn at intervals for analysis. During the course of the experiment the culture was diluted twice in growth medium containing .03 *M* D-methionine in order to maintain conditions for optimal growth. Thirty-five milliliter samples for DNA, RNA, and protein analysis were frozen in a mixture of dry ice and acetone and then stored at −30° C until such analyses could be carried out. Samples of 0.1 ml size for viable count were appropriately diluted in the growth medium for colony count. All of the cells had begun to form crescents by 20 minutes.

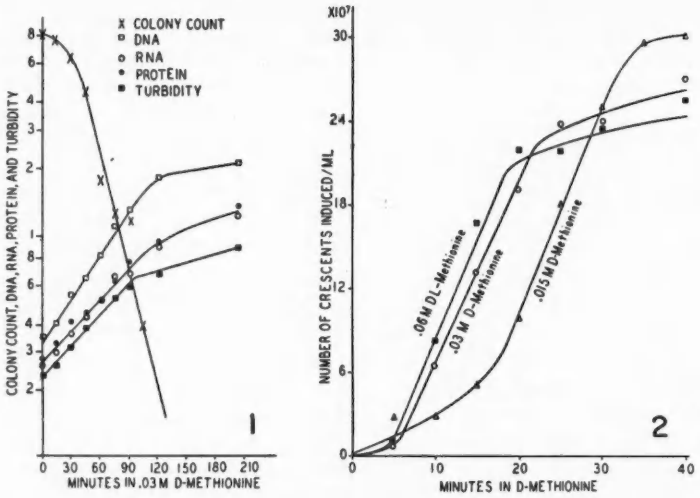

FIG. 1. DNA, RNA, and protein synthesis and loss of colony formers during transformation of *A. fecalis* LB into crescents under the influence of D-methionine. DNA by diphenylamine reaction; RNA by Orcinol reaction; protein by Lowry modification of Folin reaction. Values are relative. For absolute values see (6).

FIG. 2. Time course of crescent induction by D-methionine at different concentrations and in the presence of L-methionine. For explanation of experimental procedure see text.

In Fig. 1 various parameters of growth and reproduction are shown for a culture undergoing crescent formation in casamino acid medium in the presence of .03 *M* D-methionine.

As may be seen, the rapid loss of viability observed is not associated with a similar loss of synthetic ability. These results are similar to the effects of penicillin (8, 6). As is true in experiments with penicillin (6), the synthesis of DNA appears to be less rapidly affected than that of RNA or protein. As a result, the DNA/RNA ratio is higher in crescents than in the normal bacillary form of strain LB.

Time Course of Crescent Induction by D-*Methionine*

Induction of crescents by D-methionine was followed as a function of the length of time during which cells were in contact with D-methionine.

D-Methionine or DL-methionine was added to a culture of *A. fecalis* LB growing exponentially in L-amino acid synthetic medium which lacked methionine and aspartic acid. Samples were taken at intervals and diluted in the growth medium to a final concentration of D-methionine insufficient to induce crescent formation (.0005 *M* or eightfold below threshold). The diluted samples were incubated with aeration for 3 hours, at which time formalin was added to fix normal and crescent cells, and the crescents in each sample were counted.

The induction curves for .03 *M* and .015 *M* D-methionine and for .06 *M* DL-methionine are shown in Fig. 2. As may be seen, there is no observable

effect of L-methionine on the rate of crescent induction. Halving the concentration of D-methionine results in an increase of from 5 to 13 minutes in the lag in crescent induction which occurs prior to induction of most of the population. The latter then occurs at a rapid rate similar to that found at the higher concentration of D-amino acid. This behavior at different concentrations, similar to that observed with penicillin, suggests that the action of D-methionine in inducing a cell to become a crescent involves several steps. The larger total number of crescents formed at the lower D-methionine concentration indicates that in this case cell division persists for a longer period of time. (With a generation time of 40 minutes, an 8-minute increase in the lag could result in an increase of the population by 13%.)

Synergistic Action of Penicillin and D-*Methionine*

An experiment was carried out to test the ability of constant short-term doses of D-methionine to supplement an acting dose of penicillin in inducing crescent formation. An exponentially growing culture of *A. fecalis* LB was subjected to continuous induction of crescent formation by 40 U/ml penicillin. Aliquots of this culture were removed at intervals and subjected to short-term induction by either 200 U/ml penicillin for 4 minutes or 7.5 mg/ml D-methionine for 4 minutes. Following appropriate dilution to stop induction and subsequent incubation at 37° C, the crescents were counted.

The results of this experiment are shown in Fig. 3. As may be seen, short-term doses of D-methionine induce a larger number of crescents in the period from 5 to 40 minutes than can be expected if this agent acted completely independently of the continuous inducing action of 40 U/ml penicillin (curve M vs. MT). This behavior is similar to that of short-term doses of penicillin itself (curve P vs. PT). Penicillin and D-methionine would appear, therefore, to act synergistically in a manner suggesting that certain steps in the action of these two agents on the cell are the same.

On the other hand, some of the steps in the induction of crescent formation by D-methionine and by penicillin must be different. D-Methionine does not induce growing protoplast-like forms (globular forms) in tryptone, whereas penicillin does.[2] This may be seen from the plating efficiency of LB on tryptone and synthetic agar in the presence of D-methionine and of penicillin (Table II). Tryptone increases the concentration of D-methionine necessary for loss of viability about 2- to 4-fold, that of penicillin about 64- to 128-fold. In tryptone media containing both D-methionine and penicillin, penicillin appears to enhance rather than inhibit the ability of D-methionine to block growth. This is similar to its effect in inducing crescents in synthetic medium (Fig. 3).

Cell Wall Composition of Alcaligenes fecalis and of its Crescent Forms

The similarity of the effects of penicillin and of D-amino acids in producing crescent forms has led us to compare the amino acid composition of the cell walls of normal cells, penicillin-induced crescents, and D-amino acid induced crescents.

[2]In this medium, D-methionine produces either a non-growing crescent form or a growing bacillary form, depending upon its concentration.

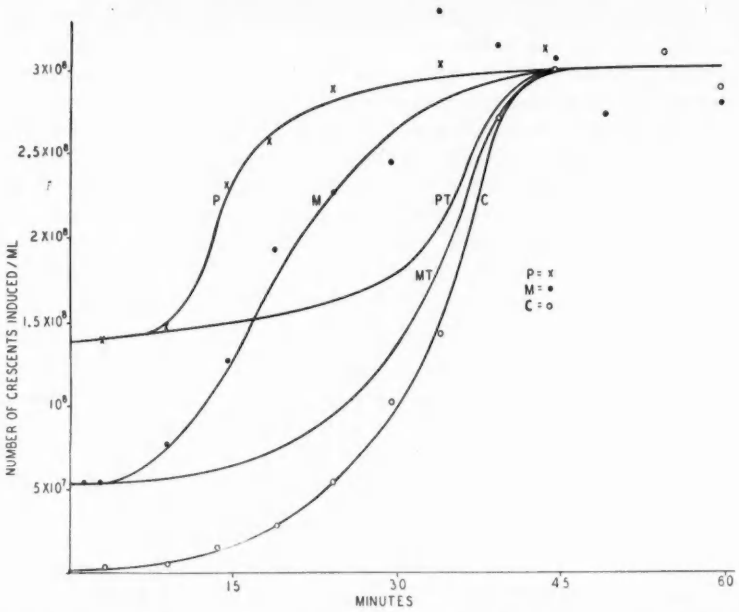

FIG. 3. Ability of D-methionine to co-operate with penicillin in inducing crescents.
C — Crescent induction by a continuous dose of 40 units/ml penicillin.
M — Crescent induction by a continuous dose of 40 units/ml penicillin with the assistance of a short-term dose of 7.5 mg/ml D-methionine for 4 minutes. Short-term doses were added to aliquots taken at the times indicated.
MT — Theoretical curve expected if penicillin and methionine act independently in inducing crescent formation.
P — Crescent induction by a continuous dose of 40 units/ml penicillin with the assistance of a short-term dose of 200 units/ml penicillin for 4 minutes. Short-term doses were added to aliquots taken at the times indicated.
PT — Theoretical curve expected if continuous dose and short-term dose of penicillin acted independently of each other.
For explanation of techniques see text as well as (8).

Cell wall preparations were made from large (5 liter) exponentially growing cultures of *A. fecalis* LB or from suspensions of crescents obtained 3 to 4 hours after adding penicillin (50 U/ml) or D-amino acids (0.015 *M*) to such exponentially growing cultures. The amino acids obtained by hydrolysis of the phenol-soluble and -insoluble cell wall fractions (see Materials and Methods) were analyzed by quantitative chromatography on columns of Amberlite IRC 50 (11).

The results of this analysis are presented in Table III. As may be seen, in the normal cell the composition of the phenol-insoluble cell wall fraction differs basically from that of the phenol-soluble fraction in that only a few amino acids are present in the former in large quantities. The insoluble fraction is composed chiefly of glutamic acid, alanine, diamino pimelic acid, and lysine. Other amino acids, such as threonine, serine, aspartic acid, arginine, etc., are present in small amounts.

TABLE II

Plating efficiency of *A. fecalis* on tryptone or synthetic medium containing penicillin and/or D-methionine

	Colonies on:				Colonies on:	
D-Methionine, mg/ml agar	Tryptone agar	Casamino acids agar	Tryptone agar + 300 U/ml penicillin	Penicillin, U/ml agar	Tryptone agar	Casamino acids agar
0	464	447	450	37	NT	476
0.14	435	423	290	75	NT	428
0.28	428	464	0	150	436	56
0.56	409	143	0	300	450	0
1.125	381	0	0	600	432	0
3.24	0	0	0	1200	452	0
4.5	0	0	0	2400	432	NT*
9	0	0	0	4800	438	NT
—	—	—	—	9600	27	NT

NOTE: A suspension of *A. fecalis* calculated to contain 450 bacteria in 0.1 ml was plated on media (tryptone, synthetic or tryptone + 300 U/ml penicillin) containing different concentrations of D-methionine and on media (tryptone or synthetic) containing different concentrations of penicillin. Plates were incubated for 4 days at 37° and the number of colonies counted.
*NT — not tried.

TABLE III

Amino acid compositions of the phenol-soluble and phenol-insoluble fractions of the cell wall of *Alcaligenes fecalis*, and of crescents of *Alcaligenes fecalis* induced with penicillin, D-methionine, or D-phenylalanine

	Phenol-soluble, μM/100 mg				Phenol-insoluble, μM/1000 mg			
		Crescents induced by:				Crescents induced by:		
	Normal cells	Pen.	D-Methionine	D-Phenylalanine	Normal cells	Pen.	D-Methionine	D-Phenylalanine
Aspartic	92	109	104	102	21.5	7.7	11.8	21.5
Threonine	53	54	45	46	13.2	3.5	5.8	9.6
Serine	55	59	48	45	11.4	3.3	6.6	9.8
Glutamic	89	91	72	93	84.9	24.5	18.4	23.4
Proline	35	36	34	33	—	—	—	—
Glycine	90	101	94	86	13.0	6.8	18.9	24.2
Alanine	91	98	95	99	135.8	16.9	10.5	19
Valine	39	42	45	42	11	—	—	—
Diamino pimelic	—	—	—	—	49.6	—	—	—
Methionine	13	7.6	11.3	7.6	—	—	—	—
Isoleucine	27	30	29	23	4.2	1.8	5.7	6.1
Leucine	69	83	90	70	16.5	4.3	14.3	14.9
Tyrosine	27	31	31	28	*	*	*	*
Phenylalanine	27	33	32	25	*	*	*	*
Lysine	58	60	58	60	44.6	—	—	—
Histidine	21	24	18	27	—	—	—	—
Arginine	55	65	65	65	27.0	—	—	—

NOTE: The methods used in the preparation and analysis of the cell wall fractions are described in the text. A dash indicates that the amounts of the amino acid if present were too small to be detected.
*In the phenol-insoluble fraction a large, broad peak was consistently found with an R_f in the vicinity of phenylalanine and tyrosine. The identity of this peak is unknown, but the possible presence of small amounts of tyrosine and phenylalanine may have been obscured by the presence of this peak.

From the data in Table III, it appears that the basic structural peptide of the phenol-insoluble cell wall fraction (see 18) contains alanine, glutamic acid, diamino pimelic acid, and lysine in the ratio 3:2:1:1.

The phenol-soluble fraction contains most of the amino acids in large quantities. Diamino pimelic acid appears to be lacking in this fraction. This fraction contains histidine, whereas the phenol-insoluble fraction does not.

It is interesting to note that those amino acids whose D-isomers are least efficient inducers of crescent formation (Table I) are present in highest quantity in the phenol-insoluble cell wall. Those which are present in low concentration or in quantities too small to detect are excellent inducers.

Cell walls of penicillin-induced and D-amino acid induced crescents are similar in composition.

The basic difference between normal and crescent cell walls lies in a reduction of the main components of the phenol-insoluble fraction, i.e., alanine, glutamic acid, diamino pimelic acid, and lysine. In addition, arginine and valine are considerably reduced.

Discussion

The effect of D-amino acids on *A. fecalis* strain LB appears to be very similar to that of penicillin. A comparison of D-methionine with penicillin is presented in Table IV. An additional point of similarity, not represented in the table, is the course of RNA, DNA, and protein synthesis during transformation by these two agents.

On the other hand, effects produced by the two agents on tryptone cultures of LB are strikingly different (Table II). Previous work with penicillin has shown that tryptone affects the eventual transformation induced rather than induction itself (8). It has been shown that D-amino acids can co-operate with penicillin to accelerate the induction process (Fig. 3). Thus, it appears that penicillin and D-amino acids may have certain steps of the induction process in common. Nevertheless, they differ in at least one step which determines

TABLE IV

Comparison of crescent induction by D-methionine and by penicillin

	Methionine	Penicillin
Morphological form in:		
Synthetic medium	Non-growing crescent	Non-growing crescent
Tryptone medium	Non-growing sphere	Multiplying globular form
Dose response	Threshold	Threshold
Threshold concentration for 100% viable colony count for:		
Synthetic/tryptone	1/4	1/64
Type of inhibition	Irreversible	Irreversible
Dynamics of induction	Multiple step	Multiple step
Competition by L-amino acids	None	None
Effect on cell wall:		
Phenol-soluble	None	None
Phenol-insoluble	Reduced alanine, glutamic acid, diamino pimelic acid, lysine, arginine, and valine	Reduced alanine, glutamic acid, diamino pimelic acid, lysine, arginine, and valine

the ability of tryptone to modify the transformation. The control over this step by D-amino acids is enhanced by the presence of penicillin (Table II) indicating that the two agents do not competitively antagonize each other. It is possible that D-amino acids may differ in their action from penicillin only by a step which involves participation in the subsequent transformation process of a growth factor present in tryptone. Recent repeated isolations in this laboratory of mutants showing ability to grow as globular forms in tryptone-containing D-methionine have indicated a high frequency of such mutants in wild-type clones. This could indicate a one-step mutation which would be consistent with the hypothesis discussed. Further study of such mutants should aid in the clarification of this aspect of the problem.

Certain characteristics of the cell wall of normal cells and crescents, irrespective of mode of induction, have been elucidated. In a study of the phenol separation of cell wall fractions of *E. coli* (16) attention was drawn to the striking similarity between the phenol-insoluble fraction of Gram-negative bacteria and the cell walls of Gram-positive organisms. A similar picture of amino acid composition has been obtained for *A. fecalis* strain LB. The cell wall of the latter differs from that of *E. coli* in having arginine present in the phenol-insoluble fraction. The phenol-insoluble cell wall fractions of both *E. coli* and *A. fecalis* differ from the cell walls of most organisms in that the former contain *both* diamino pimelic acid and lysine, whereas the cell walls of most Gram-positive and Gram-negative organisms contain only one or the other of these two amino acids. The small amounts of amino acids such as threonine, serine, aspartic acid, leucine, etc. in preparations of cell walls from Gram-positive organisms (14) and from the phenol-insoluble portion of *E. coli* (16) and *A. fecalis* strain LB cell walls may represent real components of the cell wall or only some form of contamination. The latter explanation seems unlikely for two reasons: (*a*) these amino acids are present consistently when examined by column chromatography; (*b*) the complete absence of histidine from the phenol-insoluble fraction, and of diamino pimelic acid from the phenol-soluble fraction, speaks strongly for the lack of cross contamination of the two. For these reasons, it may be justifiable to consider these "trace" amino acids as part of the cell wall of Gram-positive organisms (14, 18) and of the phenol-insoluble cell wall fraction of Gram-negative bacteria.

As has been observed with penicillin (13), treatment with D-amino acids results in a relative decrease of the main amino acid components of the phenol-insoluble fraction of the cell wall. The resulting transformation into crescents would indicate that in *A. fecalis* LB, as in *E. coli*, the phenol-insoluble fraction is responsible for the structural rigidity of the cell wall.

A consistent difference between penicillin and D-amino acid crescents lies in the amounts of the amino acids present in smaller concentrations in the phenol-insoluble fraction. The amounts of these amino acids appear to be reduced in penicillin-induced crescents and somewhat increased in ones arising from the action of D-amino acids. The significance of these differences is not clear although it is possible that they may be related to the difference in the effect of the two agents on tryptone cultures.

It is interesting that those amino acids which are lacking from or at best present in "trace" concentrations in the phenol-insoluble fraction of the cell wall are the best crescent inducers in their D-form. It is possible that these critical amino acids are catalytic in the incorporation of alanine, diamino pimelic acid, glutamic acid, lysine, arginine, and valine into the cell wall structure. It is also possible that those D-amino acids which are ineffective inducers are either racemized by the cell or are normally used in the D-form for synthetic purposes. As yet, it has not been possible to determine whether D-amino acids, which are moderately active crescent inducers (leucine, threonine), prevent their L-homologues from being incorporated into the cell wall or whether D-amino acids themselves constitute a part of the cell wall. However, it is known that D-methionine does not suppress the incorporation of intracellular methionine into the cell proteins of *A. fecalis* (9). Future work in this direction should indicate whether D-amino acid induction of crescents is due to a stoichiometric or to a catalytic effect in blocking cell wall synthesis.

It is not known whether D-amino acid induction of crescent forms is a general phenomenon. Experiments have shown that D-methionine can induce such forms in *E. coli*, but only in high concentrations (0.06 *M*). Experiments with Gram-positive organisms should prove critical in deciding this question. Regardless of the generality of the phenomenon, the results reported should afford a new approach to the study of the amino acid utilization in cell wall synthesis and in the cell division process.

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ISOTOPIC COMPETITION BETWEEN D- AND L-METHIONINE IN ALCALIGENES FECALIS[1]

K. G. LARK

Abstract

The ability of exogenous D-methionine to prevent the incorporation of exogenous L-methionine into the protein of *Alcaligenes fecalis* has been studied. D-Methionine has been found to competitively inhibit the ability of the cell to concentrate medium L-methionine with an efficiency of about 14:1. It does not inhibit the incorporation of endogenous methionine into cell protein. D-Methionine is replaceably concentrated by the cell and is able to specifically displace L-methionine previously concentrated by the cell. The significance of these findings for the mechanism of concentration of amino acids by bacteria is discussed.

Introduction

As reported in the previous paper (8) D-amino acids have been demonstrated to induce the formation of protoplast-like forms, crescents, in strain LB of *Alcaligenes fecalis*.

Investigation of the mechanism responsible for crescent induction requires a knowledge of the level at which these amino acids interact with the cell. Do D-amino acids interfere with the metabolism of their L-homologues? Do they enter into the cell? Are they racemized to the L-form? Do they enter into or interfere with protein synthesis? This investigation has sought to answer these questions.

Materials and Methods

The bacterial strain, growth media, and methods of measuring colony counts and turbidity have been described (9). L-Methionine and D-methionine were obtained from California Biochemical Foundation. The D-methionine was examined with L-amino acid oxidase for traces of the L isomer (11) and found to contain *less* than 1 μM of the L per 2 mM of the D isomer.

Separation of methionine in protein hydrolyzates was carried out by means of two-dimensional descending paper chromatography (10): solvent 1, acetic acid:butanol:H_2O (1:4:5); solvent 2, buffer (pH 8.3) saturated phenol:cresol (1:2). Methionine was oxidized with peroxide to the sulphone prior to hydrolysis of the protein. Estimation of total methionine in the eluate of the spot was carried out using the reduced ninhydrin reaction (12). This was modified to accomodate 0.1 ml of the eluate (previously treated with NaOH to remove NH_3), to which 0.05 ml of reduced ninhydrin was added, and the reaction was boiled for 15 minutes, cooled, and diluted with 0.25 ml of ethanol:H_2O, 1:1. Color was read in the Beckman DU Spectrophotometer at 570 mμ in microcuvettes.

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All radioactive counts were carried out with a Nuclear Chicago Gas Flow Counter, model D-47. In all cases thin samples were counted in which self absorption was negligible. S^{35} DL-methionine of specific activity between 25 and 50 mc/mM was obtained from the Radiochemical Center, Harwell, England. D-Methyl-C^{14}-methionine and L-methyl-C^{14}-methionine were obtained from Isotopes Specialties, Inc. Both S^{35}- and C^{14}-labeled methionines were chromatographically pure. S^{35} sulphate was obtained carrier free from Oak Ridge National Laboratories. L-S^{35}-methionine was prepared by the method of Fodor *et al.* (6) as modified by Baker and Sober (1). In so doing, all quantities were reduced proportionally to scale down the reaction to accommodate only 20–50 mg of material avoiding loss of specific activity. Purity of optically active isotopic compounds can only be estimated from the methods of preparation as less than 1% contamination of the L by the D isomer or vice versa. Insufficient material was present for carrying out direct determinations of purity.

Experimental

The Effect of D-*Amino Acids on the Rate of Incorporation of Medium Methionine into Cell Protein*

Aliquots of a culture of LB growing exponentially in L-amino acid synthetic medium (lacks methionine) were incubated with various D-amino acids for 5 minutes and then mixed with a given amount of S^{35} L-methionine. After allowing a further incubation period of 10 minutes, trichloroacetic acid (TCA) was added to a concentration of 7% and the cells were centrifuged, washed 4 times with TCA to remove medium methionine, and planchets prepared.

TABLE I

Rate of incorporation of radioactive methionine into cell protein in the presence of various D-amino acids

D-Amino acid, 0.03 *M*	S^{35} incorporated, counts/minute
Methionine	165
Valine	7240
Serine	7880
Leucine	7580
Threonine	8640
Phenylalanine	8200
—	7900

NOTE: The techniques used are described in the text. The rate of incorporation has been measured as the amount of incorporation in 10 minutes, one quarter of a generation period.

As seen in Table I, the rate of incorporation of medium L-methionine into the protein of *Alcaligenes fecalis*, LB, is lowered by the presence of D-methionine. This inhibition is not found with other D-amino acids capable of inducing crescent formation. Nevertheless, these other D-amino acids block all further *increase* in the rate of methionine incorporation into protein. This

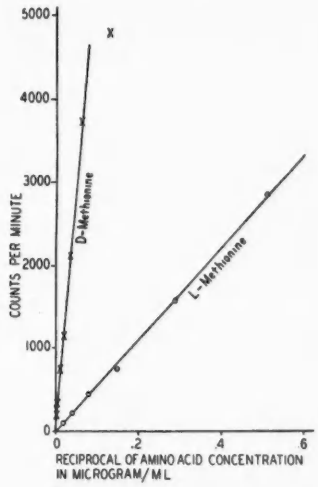

FIG. 1. Effect of D- and L-methionine concentration on incorporation of radioactive L-methionine into protein. Techniques used in this experiment are identical with those in the experiment in Table I. With no added D- or L-methionine protein activity was 5100 c.p.m.

is seen in Table II where the rate of S^{35} methionine incorporation into the protein of a normal culture and one containing D phenylalanine has been measured at different intervals of time following the addition of the D phenylalanine. As may be seen, the rate of S^{35} methionine incorporation by the D phenylalanine culture remains approximately constant and equal to the incorporation rate of the normal culture at the time of addition of the D-acid. (A similar result is obtained when S^{35} sulphate is used to label protein instead of radioactive methionine.) This indicates that during crescent formation the rate of protein synthesis remains constant.

An experiment was carried out for the purpose of comparing D- and L-methionine as inhibitors of incorporation of S^{35} L-methionine into protein (Fig. 1). The rate of S^{35} methionine incorporation in the presence of different concentrations of D- and L-methionine was tested, in the manner described for the previous two experiments. In Fig. 1, it may be seen that the difference in slope between the L- and D-methionine curves is 13.5-fold. In terms of isotope dilution this would mean that D-methionine is only 1/13.5th as efficient as L-methionine.[2]

[2]It is interesting to note that in this experiment D-methionine at infinite concentration does not completely block the incorporation of S^{35} L-methionine into cell protein. The residual radioactive L-methionine which "leaks" through has been found to be completely diluted by the addition of L-methionine. (This may be independently inferred from Fig. 2.) Furthermore, upon sonic disruption of the cells and differential centrifugation at 30,000 and then 100,000$\times g$ the distribution of radioactivity between pellets and supernatant thus obtained is the same when radioactivity is incorporated in the presence of 4 mg/ml D-methionine and when it is incorporated in the absence of diluting D-methionine. The residual activity which "leaks" through is incorporated as a linear function of time, over a period of at least 80 minutes.

TABLE II

Rate of incorporation of S^{35} methionine into cell protein of cultures growing in the presence and absence of crescent-inducing concentrations of D-phenylalanine

Time after addition of D-phenylalanine	Control		D-Phenylalanine, 5 mg/ml	
	Turb	Counts/min	Turb	Counts/min
0	.20	244	.195	185
5	.21	236	.21	203
10	.22	333	.217	240
15	.240	342	.230	254
25	.280	367	.250	246
40	.36	552	.280	243
60	.46	645	.34	237
90	.62	995	.48	240

NOTE: A 37° C exponential culture of LB was divided in half and D-phenylalanine added to one portion. Crescents appeared in the phenylalanine culture within 40 minutes. Both cultures were then further incubated at 37° C with aeration. At various intervals, aliquots of both cultures were withdrawn and incubated for 10 minutes with S^{35} methionine. These samples were then treated as in the experiment in Table I.

Mode of Competition between D- *and* L-*Methionine*

As mentioned above, it is possible to interpret the results presented as a form of isotope dilution. In this case the amount of radioactive L-methionine incorporated may be represented as:

$$x = \frac{ky}{a+b} \quad \text{or} \quad (\text{Meth-}S^{35}_{inc}) = K \frac{\text{L-meth-}S^{35}_{med}}{\frac{\text{D-meth-}S^{32}}{13.5} + \text{L-meth-}S^{32}} . \qquad (1)$$

K is a constant depending on the rate of protein synthesis and the amount of methionine used in the proteins of the organism.

Another alternative is that D-methionine causes a stoichiometric inhibition of L-methionine uptake in which the degree of inhibition is independent of the amount of L present. This may be represented as:

$$x = \frac{ky}{a} \cdot \frac{1}{b} \quad \text{or} \quad (\text{Meth-}S^{35}_{inc}) = K \frac{\text{L-meth-}S^{35}_{med}}{\text{L-meth-}S^{32}} \cdot \frac{13.5}{\text{D-meth-}S^{32}} . \qquad (2)$$

Equation 1 implies a competitive interaction between D- and L-methionine, whereas equation 2 does not.

In the experiment in Fig. 1 where the amount of (L-meth-S^{32}) is negligible compared with (D-meth-S^{32}), equation 1 becomes identical with equation 2. Differentiation between these possibilities has been made in an experiment in which aliquots of an exponentially growing culture were incubated in the presence of 100 and 350 μg/ml of D-methionine and of varying concentrations of L-methionine. After 5 minutes, a given amount of S^{35} L-methionine was added to all of the aliquots which were then incubated for 10 more minutes. The aliquots were then measured, as above, for uptake of radioactivity into the TCA-insoluble fraction. The results are presented in Fig. 2. From these results, it can be concluded that it is correct to consider the effect of D-methionine as a competitive type of isotope dilution.

The Level at which D *Competes with* L-*Methionine*

An experiment was carried out to determine whether isotope dilution occurred as a result of: (*a*) incorporation of D-methionine into the cell protein, or (*b*) inhibition by D-methionine of the ability of medium L-methionine to enter into the cell's endogenous methionine pool.

In a pilot experiment, 10-ml aliquots of culture were incubated as above with different concentrations of D-methionine, L-methionine, and a mixture of the two stereoisomers. Following 5 minutes' incubation, S^{35} sulphate was added to the culture and the mixture incubated for an additional 20 minutes. The cells were then precipitated with 7% TCA, washed free of medium sulphate, hydrolyzed with HCl, and the hydrolyzates chromatographed. Following chromatography, the cystine and methionine spots were eluted and their radioactivity counted. The results are shown in Table III.

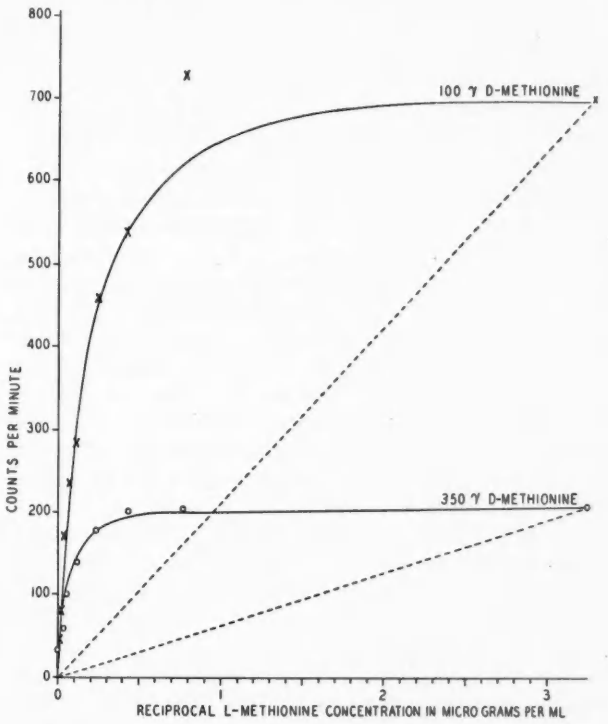

FIG. 2. Effect of non-radioactive medium D-methionine on the ability of non-radioactive medium L-methionine to dilute radioactive medium L-methionine incorporation into cell protein. For experimental techniques see text.
Solid lines, theoretical curve, equation 1, text;
broken line, theoretical curve, equation 2, text;
(S^{35} = c.p.m.) × K = 5850;
× experimental values, 100 μg/ml D-methionine;
○ experimental values, 350 μg/ml D-methionine.

TABLE III

Ratio of methionine to cystine radioactivity in protein of cells grown on S^{35} sulphate in the presence of different concentrations of D or L or D + L non-radioactive methionine

Mg/ml D-meth	M/C*	Mg/ml L-meth	M/C	Mg/ml L-meth + 2 mg/ml D-meth	M/C
2	.706	.32	.101	.32	.118
1	.816	.16	.101	.16	.174
.5	.828	.08	.119	.08	.291
.25	.955	.04	.116	.04	.432
.125	.933	.02	.140	.02	.496
.0625	1.610	.01	.167	.01	.580
0	1.55	0	1.23		

NOTE: Spots on chromatograms were located by means of autoradiography and the entire spot eluted. Location and elution of spots of low activity (L-meth) were subject to large error due to incomplete delineation of spots on radioautographs. In this experiment one-dimensional acetic acid:butanol:H_2O chromatography was used. Concentration of methionine was too low to allow measurement of specific activities. Methionine to cystine ratios were measured on the assumption that added medium methionine does not interfere with cystine synthesis. Neither cystein not cystine was present in this growth medium.

*M/C = ratio of methionine S^{35}/cystine S^{35}.

As may be seen, the amount of isotope dilution found with the D alone is small compared with that of a 10-fold lower concentration of L. Of greater significance, however, is the inability to obtain effective isotope dilution with non-radioactive L-methionine in the presence of 10-fold greater concentrations of D.

Due to the large number of samples in this experiment, hydrolyzates were separated in one dimension only. Specific activities were not measured. Large amounts of non-specific radioactive contamination of low-activity spots could be only approximated by subtracting radioactivity of spots of identical area taken from other positions on the chromatograms. In addition, spots of low radioactivity were not clearly delineated. This led to a consistent positive error in determining methionine/cystine ratios in those preparations with low counts in the methionine samples as well as a general error of $\pm 50\%$ in all count determinations.

For this reason, a more limited but more quantitative experiment was carried out in which the specific activity of methionine was measured instead of comparing the methionine/cystine ratios. This experiment and its results are described in Table IV.

The results of this experiment are similar to those in Table III. A comparison of the specific activity of protein in the presence of D and L and of only L, or only D, non-radioactive methionine demonstrates that D-methionine interferes with the entry of medium methionine into protein but not with the incorporation of endogenously synthesized methionine. The difference in specific activity of protein methionine synthesized in the presence of 2 mg/ml D- and .04 mg/ml L-methionine, as compared with .04 mg/ml L-methionine alone, is 953 as compared with 227. Inasmuch as increasing the L-methionine concentration to .08 or .16 mg/ml results in a stoichiometric decrease in

TABLE IV

Specific activity of protein methionine from cells grown on S^{35} sulphate in the presence of D or L or D + L non-radioactive methionine

Medium D-meth conc.	Medium L-meth conc.	Protein methionine		
		Ninhydrin	Counts/minute	Sp. act.
0	0	.395	3540	8980
0	0	.385	3180	8260
0	.02 mg/ml	.935	460	492
0	.04 mg/ml	.530	114	215
0	.04 mg/ml	.330	79	240
0	.08 mg/ml	2.75	305	111
0	.16 mg/ml	1.165	70	60
2 mg/ml	0	.320	2210	6900
2 mg/ml	0	.472	2980	6320
2 mg/ml	.04 mg/ml	.425	415	976
2 mg/ml	.04 mg/ml	.462	430	931

NOTE: Techniques used in this experiment were similar to those in the previous experiment with the following differences: *100*-ml aliquots were used and the methionine was separated by two-dimensional chromatography. Ninhydrin estimation is discussed in Materials and Methods. Total elution of spots was unnecessary, as specific activity was measured.

It should be remembered that with a generation time of 40 minutes, only 28% of the total protein methionine has been incorporated during the 20-minute incubation with radioactive sulphate.

specific activity, this 4.2-fold *increase* in specific activity in the presence of 2 mg/ml D-methionine represents a 4.2-fold *decrease* in the ability of medium L-methionine to enter the cell's protein.

The stoichiometric decrease in specific activity of protein methionine derived from S^{35} sulphate as a result of increasing the medium non-radioactive L-methionine may be represented as:

$$\text{specific activity} = \frac{K}{(\text{L-meth})_{\text{effective}}}. \tag{3a}$$

From the data in Fig. 2 and from equation 1 the effective L-methionine concentration in the presence of D-methionine can be represented as:

$$(\text{L-meth})_{\text{eff}} = \text{L-meth}_{\text{med}} \left(\frac{\text{L-meth}_{\text{med}}}{\text{L-meth}_{\text{med}} + \frac{\text{D-meth}_{\text{med}}}{a}} \right), \tag{3b}$$

where a is the efficiency constant for the interaction of D- with L-methionine, found to be 13.5 in the experiment in Fig. 1.

Combining equations *3a* and *3b* one finds that:

$$\frac{(\text{Spec. act.})_{\text{L+D}}}{(\text{Spec. act.})_{\text{L}}} = 1 + \frac{(\text{D-meth})_{\text{med}}}{a(\text{L-meth})_{\text{med}}} \tag{3}$$

or

$$4.2 = 1 + \frac{2}{a(.04)},$$

$$a = 15.6.$$

This value for a is in good agreement with the value of 13.5 obtained from Fig. 1.

From these data, it follows that the primary effect of D-methionine is to inhibit the entry of exogenous L-methionine into the pool of amino acids used by the cell for protein synthesis. If D-methionine is used in protein synthesis in place of the L isomer the data in Table IV indicates that it must be at least 100-fold less efficient than the latter. These data exclude the possibility that an appreciable amount of D-methionine is racemized to the L-form which then should enter into protein synthesis.

Ability of D-*Methionine to Enter Specifically into the Methionine Concentrating System of A. fecalis, LB*

A. fecalis LB concentrates L-methionine despite the presence of other L-amino acids in the medium. The following experiment demonstrates that this concentrated L-methionine can be replaced by new medium methionine up to a certain extent. An exponentially growing culture of LB in methionine-free medium (L-amino acid medium) was incubated at 37° with chloramphenicol, 75 μg/ml, for 20 minutes. (This blocks incorporation of methionine into TCA-insoluble material to an extent of 99%, allowing methionine to remain concentrated in the amino acid pool of the cells.) S^{35} L-methionine was then added to the chloramphenicol-treated culture at a concentration of 0.1 μg/ml (2300 counts/min/ml). Five, ten, or forty minutes after the addition of radioactive methionine, one set of 10-ml aliquots, A (not replaced), was removed, centrifuged for 10 minutes in the cold at 30,000×*g*; the pellets were extracted with H_2O at 100° C for 5 minutes and again centrifuged. An aliquot of the resulting supernatant was counted. Another set of 10-ml aliquots, B (replaced), was simultaneously, i.e. at 5, 10, or 40 minutes, mixed with non-radioactive L-methionine to a concentration of 1 mg/ml and further incubated at 37° for 10 minutes. These aliquots

TABLE V

Replaceable and non-replaceable concentration of S^{35} L-methionine during different time intervals

Minutes after adding S^{35} L-methionine	A, not replaced	B, replaced	(A−B)
5	447	71.3	376
10	498	127	371
40	718	356	362

NOTE: A total of 1.4×10^8 bacteria/ml S^{35} L-methionine was added at 0 minutes to a concentration of .1 μg/ml (2500 c.p.m.). Techniques used in this experiment are described in text.

were then treated as the others. In Table V it may be seen that the non-replaceable fraction of concentrated methionine (B) increases with time whereas the replaceable fraction (A−B) does not. Chromatographic analysis of the non-replaceable fraction has shown that it is not all in the form of free methionine. The replaceable fraction *is* chromatographically identical with methionine.

Various D-amino acids have been tested for their ability to block concentration of radioactive L-methionine within the cell. Aliquots of a chloramphenicol-treated culture were mixed with various D-amino acids or dinitrophenol and incubated for 5 minutes with radioactive S^{35} L-methionine. After incubation, the samples were chilled and centrifuged at 0° for 5 minutes at 30,000×*g*. The pellets were extracted with boiling water and again centrifuged. The resulting supernatants were examined for radioactivity (Table VI). As in *E. coli* (4), the concentration of L-methionine is blocked by dinitrophenol. Of the D-amino acids capable of inducing crescent formation, only D-methionine inhibited the concentration of L-methionine.

TABLE VI

Concentration of S^{35} L-methionine in the presence of various non-radioactive D-amino acids and of dinitrophenol (DNP)

.03 *M* D-amino acid	S^{35} methionine concentrated, counts/minute
Methionine	195
Valine	1330
Serine	1185
Leucine	1320
Threonine	1155
—	1358
DNP (10^{-3} *M*)	224

NOTE: Techniques used in this experiment are described in the text. Medium concentration of S^{35} L-methionine was 3 μg/ml or 25,000 counts/min/ml. Bacterial concentration = 4×10^8 cells/ml.

D-Methionine has also been tested for its ability to replace previously concentrated radioactive S^{35} methionine. A culture was allowed to concentrate radioactive S^{35} L-methionine under the conditions described above. Aliquots of this culture were then further incubated with various concentrations of D- or L-methionine for an additional 5 minutes. Incubation of the control was carried out with no added D- or L-methionine. The techniques used in this experiment were essentially those used in Table V. The results are shown in Table VII, where the per cent replaced methionine is calculated from data of the type shown in Table V and represents (A−B)/A.

In Table VII it may be seen that both L- and D-methionine replace previously concentrated L-methionine to a total extent of approximately 75%. However, at concentrations at which submaximal replacement occurs, the L-enantiomorph appears to be much more efficient.[3]

[3]A quantitative comparison of the ability of D- and L-methionine to replace previously concentrated L-methionine is not possible since a further increase in the non-replaceable fraction occurs during the replacement period. This increase, negligible at high concentrations (1000 μg/ml), may be considerable at lower concentrations, and will vary with both the medium methionine concentration and the specific activity of the medium methionine.

TABLE VII

Ability of D-methionine to replace previously concentrated L-methionine

Meth.	Medium μg/ml	% replaced
0 added	.05	0
L added	.21	4
	.37	51
	.69	70
	1.33	74
	1000	77
D added	1.6	22
	3.2	38
	12.8	56
	51.6	69
	1000	73

NOTE: Techniques used in this experiment are described in text.

Concentration of Replaceable D- *and* L-*Radioactive Methionine with Increasing Amounts of Medium Methionine*

Twenty-ml aliquots of a chloramphenicol (75 μg/ml) treated culture of LB, 3.3×10^8 cells/ml, were incubated for 5 minutes in the presence of varying amounts (.05–4 μg/ml) of radioactive D, or L, methionine. Following incubation, 10 ml was immediately centrifuged at 4° C and analyzed for the amount of concentrated amino acid. The supernatant was also analyzed for the concentration of medium methionine.[4] The remaining 10 ml was further incubated for 10 minutes in the presence of 1 mg/ml non-radioactive methionine. These samples were then also examined for residual concentrated methionine. The difference between the two 10-ml samples yielded the amount of replaceable methionine concentrated. It will be seen that the construction of this experiment is essentially the same as that used for the 5-minute sample in Table V. The results are presented in Fig. 3 where the ordinate represents replaceable methionine calculated as (A − B) in Table V.

As may be seen in Fig. 3, both the D and L stereoisomers are concentrated replaceably by LB. However, the maximum amount of amino acid concentrated is approximately 2.5 times greater for the L than for the D enantiomorph. *Both* D *and* L *stereoisomers are concentrated with equal efficiency at medium concentrations below .2 μg/ml.* As a result, the concentration of medium methionine necessary to allow the cell to accumulate one-half the maximum amount of methionine which the cell is capable of accumulating

[4]It is important to measure the medium methionine directly, since appreciable quantities are removed by the cells at low medium methionine concentrations. Following replacement (aliquot B in Table V), it has been found that almost all of the medium radioactivity is restored.

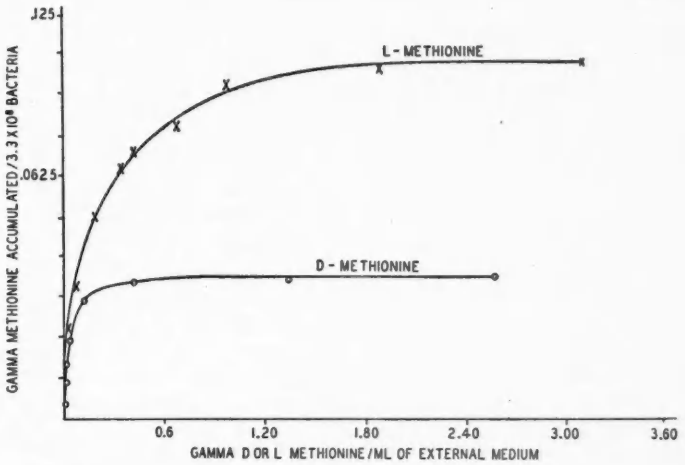

FIG. 3. Effect of medium D- or L-methionine concentration on the respective amounts of replaceable D- or L-methionine accumulated by the cell. The experimental techniques are described in the text and in Table V. Ordinate values were obtained as were (A−B) in Table V and converted to μg of methionine.

Maximum replaceable L-methionine accumulated per cell = 1.32×10^6 molecules,
maximum replaceable D-methionine accumulated per cell = 5.05×10^5 molecules.
L-Methionine medium concentration for half-saturation = .168 μg/ml,
D-methionine medium concentration for half-saturation = .043 μg/ml.

(medium concentration for half-saturation) is considerably lower for D-methionine than for L-methionine. On the simple assumption that methionine is accumulated within the cell by combining reversibly with specific sites,

$$K = \frac{(M)\ \ (S)}{(M-S)} \tag{4}$$

where (M) = medium methionine concentration,
(S) = free site concentration,
$(M-S)$ = combined site concentration.

The medium concentration (M) at half saturation, where $(S) = (M-S)$, is the value of K.

The experiment in Fig. 3 was carried out using C^{14} methyl labeled D- and L-methionine in L-amino acid medium (which lacks methionine). Identical results were obtained when the experiment was carried out using this form of isotopically labeled methionine and a growth medium in which all amino acids were replaced by ammonia as a nitrogen source. Experiments with S^{35}-labeled L-methionine yield results identical with those shown in Fig. 3 for C^{14}-labeled L-methionine. A limited experiment with S^{35} D-methionine yields an identical value for the total replaceable methionine accumulated and indicates a medium half saturation value of less than .08 μg/ml.

Discussion

Methionine was chosen for these experiments because it is an efficient inducer of crescent formation, and it is a convenient material from the point of view of availability of isotope labeling and of non-radioactive stereoisomers of high optical purity. The following points have been established: (*a*) D-Methionine specifically blocks entry of radioactive L-methionine into cell protein with a stoichiometric efficiency of about 14:1. (*b*) In doing this, D-methionine behaves competitively, the degree of blockage depending on the D to L isomer ratio. (*c*) D-Methionine does not block the entry of endogenous methionine into cell protein, but interferes with the entry and concentration of medium L-methionine into the cell. (*d*) D-Methionine can replace L-methionine previously concentrated by the cell and is itself concentrated replaceably within the cell. (*e*) In the presence of chloramphenicol, D-methionine is concentrated by the cell to a saturation level equal to approximately 0.4 of that obtained with L. The medium concentration at which the cell is one-half saturated with D is about 1/4 that at which it is one-half saturated with L.

Previous experiments with *Escherichia coli* (4) demonstrated that replaceable concentration of amino acids by this bacterium was stereospecific for the L-form of the amino acid. In addition, concentration of L-methionine was shown to be independent of the presence of other structurally unrelated L-amino acids (4, 2). In the case of *A. fecalis* LB, concentration of methionine is not stereospecific. On the other hand, it is independent of the presence of other D- and L-amino acids. (The presence of as much as 1000 μg/ml L-glutamic acid in the growth medium has no effect on L- or D-methionine concentration.) Both D and L medium methionine are capable of replacing either D- or L-methionine previously concentrated. From this, it seems logical to assume that D- and L-methionine are concentrated within the cell by the same methionine specific system. The two stereoisomers differ, however, in the parameters of their concentration, as outlined in (*e*) above.

Cohen and Monod (3) have discussed two hypotheses accounting for the concentration of metabolites by the bacterial cell: (*a*) specific enzymatic concentration of metabolites ("permease" hypothesis) and (*b*) combination of metabolites with specific sites within the cell as represented in equation 4 above (stoichiometric hypothesis). In distinguishing between these alternatives, it was noted that for compounds which utilize the same specific concentrating mechanism, a decrease in the one-half saturation medium concentration (dissociation constant in hypothesis (*b*)) should not be accompanied by a decrease in the maximum amount of metabolite which the cell may concentrate if the stoichiometric hypothesis (*b*) is correct. It is impossible therefore to explain the results obtained above with D- and L-methionine on the basis of a simple competition for a common binding site within the cell. These results would therefore seem to support the "permease" hypothesis.

On the other hand, results obtained by measurement of isotope dilution during protein formation show that D-methionine is 1/14th as efficient as L in preventing radioactive medium L-methionine from entering protein. The stoichiometric inhibition by medium L-methionine of incorporation of S^{35} sulphate into protein methionine indicates that a saturation level of concentrated methionine is not reached in growing cells at medium methionine concentrations of .16 mg/ml. On the other hand, in the presence of chloramphenicol, saturation is reached at a medium concentration of 2 μg/ml. Furthermore, the methionine formed endogenously from sulphate is not affected by the presence of exogenous D-methionine, which can, nevertheless, replace exogenous L-methionine concentrated in the presence of chloramphenicol. It would appear from this that the ability of the cell to concentrate methionine in the presence of chloramphenicol is not necessarily related to its ability to concentrate exogenous methionine for use in the synthesis of cell protein.

These results are compatible with the hypothesis proposed by Cowie and McClure (5) of two amino acid pools within the cell, the expandable and the internal pool. According to this hypothesis, L-methionine upon entering the cell should enter the expandable amino acid pool, from which it can be competitively displaced into the medium by D-methionine. It then could pass into the internal amino acid pool (of fixed size) in which it can be no longer affected by D-methionine. This latter pool may also be fed by endogenously synthesized methionine, whose rate of entrance into this pool (perhaps synthesis) is determined by the methionine requirements of the pool.[5] The rate at which the requirements of the internal pool may be met from the expandable pool is proportional to the amount of methionine in the expandable pool, whose size is controlled by the medium methionine concentration. The over-all efficiency with which D-methionine blocks the incorporation of L-methionine into protein should thus be a function of its ability to competitively block the entry of the latter into the internal pool rather than its effect on the expandable pool alone (as measured in the experiments with chloramphenicol-treated cells).

It is interesting to speculate on the nature of an amino acid transporting enzyme which, although specific for a given amino acid, is not stereospecific. This would imply that if an amino or carboxyl group is activated in the transport process, activation is *not* involved in the same step which confers specificity on the transport reaction. Inversion of the molecule would thus cause no interference with the reaction, since steric recognition of the terminal grouping of the amino acid molecule and of either the amino or carboxyl group would occur separately.

The concept of two distinct reactions has been proposed by Britten *et al.* (2), who suggested that the activation step was one in which some site within the cell was activated to accept the entering amino acid. However, as

[5]It appears likely that the entry of endogenous methionine into such an internal pool would be controlled by some type of feedback mechanism.

mentioned above, it is difficult to reconcile such a site hypothesis with the observed parameters of D- and L-methionine concentration.

It is not clear whether the process of amino acid concentration by the cell is affected by chloramphenicol. If it is, a differential effect of chloramphenicol on D- and L-amino acid concentration might also be suspected to occur, since previous work has shown that the D and L threo isomers of chloramphenicol will inhibit peptide formation from L- and D-amino acids respectively (7).

The form in which methionine is bound in the irreplaceable fraction which is extractable by water at 100° is not known. However, we have found that both D- and L-methionine appear to an equal extent in this fraction. Experiments to determine whether or not D-methionine is incorporated into protein have so far proved inconclusive. Further experiments employing isomers of chloramphenicol should serve to clarify these questions.

The data obtained have not enabled us to arrive at any conclusions as to the mechanism of crescent formation by D-amino acids. It appears that the action of these compounds may be exerted from within the cell. An interesting possibility lies in the hypothesis of two amino acid pools—the expandable amino acid pool capable of exchange with the medium and the internal pool incapable of such exchange. It is possible that cell wall synthesis is dependent on the presence of certain amino acids, such as L-methionine, in the expandable pool. The use of such amino acids for cell wall synthesis could be interfered with by the presence in this pool of large concentrations of the D isomer. On the other hand, protein synthesis using methionine from the internal pool could proceed without interference.

Whether or not this proves to be the case, the fact that D-amino acids interfere with synthesis of the phenol-insoluble fraction of the cell wall (8) but not with the synthesis of most cell proteins indicates that there may be a connection between this cell wall fraction, apparently responsible for cell wall structure, and the amino acid concentrating mechanism of the cell.

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STUDIES ON THE ANTIGENIC STRUCTURE OF TRICHINELLA SPIRALIS LARVAE[1]

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Abstract

By means of physical and chemical procedures seven antigenic fractions have been isolated from *Trichinella spiralis* larvae, as determined by complement fixation. Some evidence is presented to indicate that at least some of the isolated fractions are antigenically distinct and that antibodies in *Trichinella*-infested rabbit develop at different times for different fractions. Importance of using a properly selected antigen in serodiagnosis of trichinosis is discussed.

Introduction

Serology has been employed in the diagnosis of trichinosis since 1911 when Ströbel (7) demonstrated the presence of complement-fixing antibodies in the sera of *Trichinella*-infected animals. No attempt, however, with the exception of the work of Melcher (6), has been made to determine the antigenic structure of *Trichinella spiralis* larvae. Melcher isolated six fractions: (1) polysaccharide, (2) lipoids, (3) defatted residue insoluble at pH 8.3, (4) alkaline extract, (5) acid-insoluble protein fraction, and (6) acid-soluble protein fraction. Antigenicity of the fractions was evaluated by a precipitin test. Lipoid and defatted fractions were found to be unsuitable for the test; the polysaccharide and the acid-soluble protein fractions displayed a high degree of antigenicity. Antigenicity of the alkaline extract, which was an earlier stage in the extraction of the acid-soluble fraction, most likely was due to the presence of the latter fraction. The acid-insoluble fraction failed to show any reactivity.

The present communication deals with a more detailed study of the antigenic structure of the larvae of *T. spiralis*. The investigation was directed mainly towards the isolation of an antigenic fraction suitable for the complement fixation test. The work resulted in the isolation of seven antigenically distinct fractions. The antigenicity of each fraction was evaluated by the complement fixation test. Each fraction was also analyzed chemically.

Materials and Methods

T. spiralis Larvae

The strain of *T. spiralis* employed in this study was isolated by one of us (E. K.) from a polar bear in 1953. It was maintained in the laboratory by serial passage in rats and rabbits. The larvae for this work were obtained from rats, each of which had been infected by mouth with approximately

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2000 live larvae. The larvae were purified in the usual manner by digesting the skinned ground carcasses of infected rats with pepsin in the presence of HCl and subsequently washing the larvae through a stainless steel screen.

Procedures of Fractionation

The procedure of fractionation employed was a modification of that reported earlier in connection with *Histoplasma capsulatum* (Labzoffsky *et al.* (5)). The steps of fractionation were carried out at room temperature unless otherwise stated and are outlined in Fig. 1. Sterile precautions were taken whenever practicable.

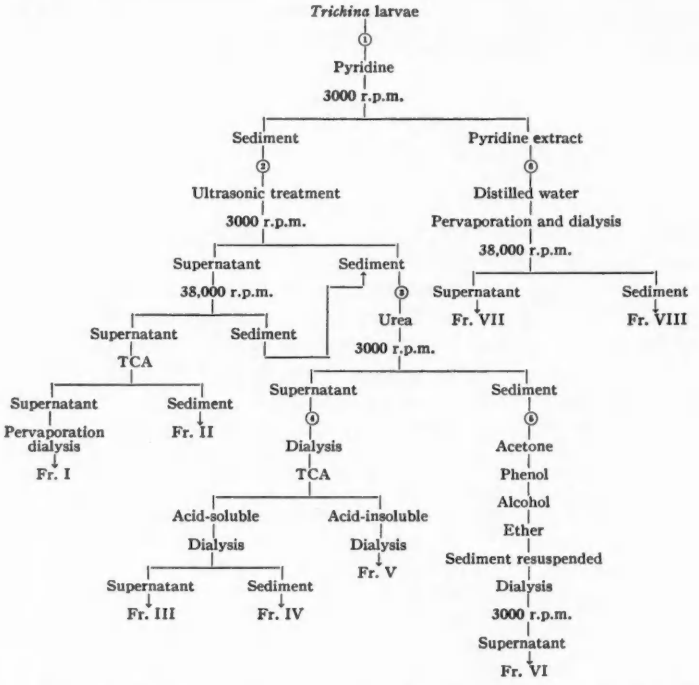

FIG. 1. Flow diagram of basic procedures of fractionation of *T. spiralis* larvae.

Step 1.—Freeze-dried larvae were extracted with approximately 20 times their volume of pyridine. The materials were left in contact for 2½ hours with frequent shaking. The purpose of the treatment with pyridine was to extract lipids and pyridine-soluble antigen and also to "unmask" the antigenic architecture of the organism. For this purpose pyridine appears to be very well suited, since it has no apparent deleterious effect on the proteins or antigens, providing the time of contact is carefully controlled. At the end of the 2½ hours of contact the materials were centrifuged and both the sediment and the pyridine extract saved.

Step 2.—The sediment was resuspended in distilled water and exposed to six periods of ultrasonic vibration, each period being of 2 hours' duration.* After each period of vibration the material was centrifuged at 3000 r.p.m. for 20 minutes. The sediment was resuspended to the original volume in distilled water and the supernatant saved. After the last vibration the sediment was saved for step 3. The six supernatants were pooled and centrifuged at 38,000 r.p.m. in Spinco model L centrifuge. The sediment from this operation was added to the original sediment for step 3 and the supernatant was precipitated with 10% trichloracetic acid. The acid-soluble supernatant was separated by centrifugation and marked fraction I and the acid-insoluble sediment was marked fraction II. Both fractions were dialyzed against tap water. Fraction I was concentrated to about one third of its original volume by pervaporation in front of an electric fan while it was still in the dialyzing bag.

Step 3.—To the wet-pooled sediment from step 2 an equal volume of urea crystals was added and the material was left in a reciprocating shaker-bath at 37° C for 48 hours. At the end of that period an equal volume of distilled water was added and the material returned to the bath for an additional 24 hours. It was then centrifuged, the sediment saved for step 5, and the supernatant treated as described below in step 4.

Step 4.—The supernatant was dialyzed against tap water for 48 hours and then precipitated with 10% TCA. The acid-insoluble fraction was resuspended in buffer, pH 7.2,† and designated fraction V. This and the acid-soluble material both were dialyzed against tap water for 48 hours. During dialysis a copious precipitate was formed in the acid-soluble material. This was removed by centrifugation, redissolved in buffer, and marked fraction IV, the supernatant fraction III. It must be mentioned that fraction IV did not separate regularly. There were several different batches of larvae from which fraction IV could not be obtained. At the moment the writers are at a loss to explain this irregular behavior.

Step 5.—The sediment from step 3 was washed twice with pure acetone and once with 80% acetone. After the last centrifugation the residue was dried in front of an electric fan. The dark-gray residue was next pulverized, mixed with two volumes of 80% phenol, and left in the 48° C water bath for 30 minutes with frequent stirring. The phenol extract, after centrifugation, was discarded and the sediment treated with absolute alcohol to remove the last traces of phenol. After removal of alcohol the sediment was extracted with ether, centrifuged, and then air-dried. The entire cycle of phenol, alcohol, and ether treatment was repeated two more times. Centrifugation in each case was performed at 3000 r.p.m. for 30 minutes. The dry sediment, obtained after the last ether extraction, was resuspended in saline, dialyzed for 48 hours against saline, and centrifuged. The opalescent supernatant was marked fraction VI-polysaccharide.

*The Ultrasonic generator employed was manufactured according to our specifications by Conway Electronic Enterprises Ltd., Toronto. It has a frequency of 1000 kc and delivers to the crystals 1000 acoustic watts and is water-cooled. The treatment vessel with cooling jacket has been described earlier (1).

†Concentration of phosphate buffer used throughout this experiment was 0.02 *M.*

TABLE I

Titration of fractions prepared for *T. spiralis* larvae against immune rabbit serum

	Antigen 1 in:								
	8	16	32	64	128	256	512	1024	2048
Fraction I	++++	++++	++++	++++	++++	+++	++	—	—
Fraction II	++++	++++	++++	+++	++	—	—	—	—
Fraction III	++++	++++	++++	++++	++++	+++	++	—	—
Fraction IV	++++	++++	++++	++++	++++	++++	++	—	—
Fraction V	++++	++++	++++	++++	++++	++++	++++	++++	+++
Fraction VI	++++	++++	++++	++++	++	—	—	—	—
Fraction VII	++++	++++	+++	++	—	—	—	—	—
Fraction VIII	—	—	—	—	—	—			

NOTE: Immune serum 1:50.

Step 6.—The pyridine extract from step 1 was diluted with an equal volume of distilled water and transferred to a dialyzing bag. Dialysis and pervaporation of this material were accomplished simultaneously by half-submerging the bag in a container of running tap water in front of an electric fan. This process was continued until the volume was reduced to approximately one-tenth of the original volume of pyridine extract. The material was then centrifuged at 38,000 r.p.m. for 90 minutes. The resultant supernatant was marked fraction VII and the sediment resuspended in buffered saline fraction VIII.

Sera

Only experimental sera, prepared in rabbits, were used in this work. These animals were each infected by mouth with approximately 2000 live larvae. Samples of blood were collected at weekly intervals. All sera were kept frozen at −50° C and for the test were inactivated at 60° C for 30 minutes. All serum samples, including preimmunization sample from each animal, were titrated simultaneously.

Complement Fixation Test

The technique of this test was described earlier (Labzoffsky *et al.* (4)), the only modification being overnight incubation at 4° C instead of 75 minutes at 37° C. Two units of antigen were used in the test. The unitage was determined by titration of a given antigen against two units of antiserum. One unit of either reagent was defined as the highest dilution that gave complete (4+) fixation. When comparative titrations were made, all serum samples from the same animal were always titrated simultaneously. The usual controls were always included in each test and the tests were read after serum and antigen controls were completely clear, usually 20 minutes after addition of haemolytic system. Positive controls in all cases gave complete (4+) fixation. For the sake of brevity, controls are omitted from the tables.

Ultraviolet Absorption Spectra

Measurements were carried out on a Beckman model DU spectrophotometer, equipped with photomultiplier attachment, with the spectrum of the hydrogen tube. All fractions were diluted with phosphate buffer, pH 7, to contain equivalent antigenic units, thus giving comparative optical densities.

Experimental Results

Seven of the eight fractions isolated were found to be antigenically active as determined by complement fixation. The results of the titration of these fractions are summarized in Table I.

As is seen from this table fraction VIII was found to be non-antigenic. The difference in the titers of the rest of the fractions could be, on the whole, attributed to the degree of concentration rather than to the proportion present in the original material.

None of the fractions were found to be anticomplementary even in the lowest dilution tested (1:8).

All fractions with the exception of fraction II were soluble, all were heat-stable, withstanding boiling for 5 minutes without losing reactivity. All fractions were found to be stable at 4° C for at least several months and at −20° C for at least 2 years.

An attempt has been made to differentiate these fractions serologically by titrating them against the trial bleedings collected at weekly intervals from infected rabbits. Series of titrations of successive sera from a number of rabbits against the various fractions were prepared. All were similar, and the results of the titrations of sera from one of the rabbits are recorded in Table II.

TABLE II

Simultaneous titration of trial bleedings from rabbit No. 1600 against fractions of *T. spiralis* larvae

		Serum 1 in:					
		8	16	32	64	128	256
Bled 1 week after infection	Fraction I	—	—	—	—		
	II	—	—	—	—		
	III	—	—	—	—		
	IV	—	—	—	—		
	V	++++	++++	+++	++		
	VI	—	—	—	—		
	VII	—	—	—	—		
Bled 2 weeks after infection	Fraction I	—	—	—	—		
	II	+++	+	—	—		
	III	—	—	—	—		
	IV	—	—	—	—		
	V	++++	++++	++++	++++	+	
	VI	+++	++	—	—		
	VII	—	—	—	—		
Bled 3 weeks after infection	Fraction I	—	—	—	—		
	II	++++	+++	+	—		
	III	—	—	—	—		
	IV	+++	+	—	—		
	V	++++	++++	++++	++++	++	—
	VI	++++	++	—	—		
	VII	—	—	—	—		
Bled 5 weeks after infection	Fraction I	++++	++++	++++	+	—	
	II	++++	++++	++++	++++	++++	++
	III	++++	++++	++++	++	+	—
	IV	++++	++++	++++	++++	++++	+
	V	++++	++++	++++	++++	++++	++++
	VI	++++	++++	+++	+	—	
	VII	++++	++	—	—	—	
Bled 6 weeks after infection	Fraction I	++++	++++	++++	++++	++	—
	II	++++	++++	++++	++++	++++	+
	III	++++	++++	++++	++++	++	+
	IV	++++	++++	++++	++++	++++	++
	V	++++	++++	++++	++++	++++	++++
	VI	++++	++++	++++	++++	+++	++
	VII	++++	++++	++++	++++	++	—

NOTE: Each antigen was diluted to contain two antigenic units. Preimmunization serum sample failed to react with any of the fractions and for simplicity the results are omitted from this table.

Unfortunately trial bleedings 4 weeks after infection were not available from the infected rabbits and therefore some information has been missed. However, it will be noted that at the end of 1st week antibodies against fraction V only have appeared; at the 2nd week appeared those against fractions II and VI as well as fraction V. At the 3rd week antibodies to fraction IV and at the 5th week antibodies against all the fractions are present, with increases in the titer of the later appearing antibodies at the 6th week.

From the table it would appear that fraction V is different from the others, since antibodies to it appear earliest. Fractions II and VI are similar in time of appearance but differ in that fraction II is an acid-insoluble protein and fraction VI is a polysaccharide. Antibodies against fractions III and VII appear at the 5th week; fraction VII is, however, at a lower titer than fractions III or I and, moreover, differs from them in being pyridine-soluble. As will be seen later fractions III and I differ in their ultraviolet absorption spectra.

An attempt has also been made to differentiate these fractions by ultraviolet absorption tests, the results of which are illustrated in Fig. 2. From these curves it is apparent that fractions I and VI differ from each other and also from the rest of the fractions. Fraction I obviously is not pure and consists of at least two components as indicated by two broad maxima. Fraction II did not give a satisfactory enough curve for interpretation, possibly because this fraction was not soluble.

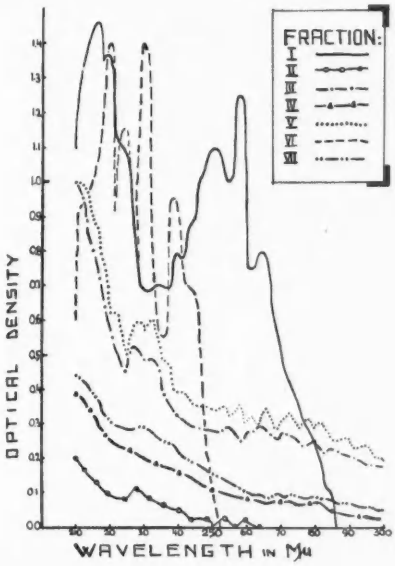

FIG. 2. Ultraviolet absorption spectra of antigenic fractions of *T. spiralis* larvae.

There seems to be an apparent similarity of curves obtained with fractions III and V. However, they differ in their chemical properties, fraction III being acid-soluble and fraction V acid-insoluble. Further, antiserum produced against fraction III failed to react with fraction V and conversely antiserum for fraction V did not react with fraction III. Fractions IV and VII curves, although appearing to have similar slope, possess different properties. Fraction IV was shown to be pyridine-insoluble whereas fraction VII was shown to be soluble in pyridine.

The chemical analysis of the fractions is summarized in Table III, which gives some information as to the general composition of the different antigenic fractions as well as the differences in their structure. Fraction I appears to be a glycoprotein complex and differs from all the other fractions. Fraction II is also glycoprotein with strong reaction for amino acids and reducing sugars. Fractions III and IV differ in their amino acid composition. Fractions IV and V are both of glycoprotein nature, but fraction IV has more of the protein character whereas fraction V possesses more carbohydrate characteristics. Fractions VI and VII are both of polysaccharide nature but not identical.

A pilot test indicated that all seven fractions produced allergic reactions (skin test) when inoculated intradermally into a *Trichinella*-infected rabbit. Only one dilution of each fraction was used representing one complement-fixing unit. No such reactions were observed in the skin of a normal rabbit.

From the practical standpoint it was deemed desirable to compare one of the commonly used antigens with our fractions. This saline-extracted antigen was prepared as outlined by Witebsky *et al.* (7) and the same bleeding

TABLE III

Chemical spot analysis of the fractions of *T. spiralis* larvae

Test	Fraction I	II	III	IV	V	VI	VII	Remarks
Sakaguchi (arginine)	—	+	—	++	++	—	—	[1] Reddish
Adamkiewicz (tryptophane)	—	+	+	++	+	—	—	[2] Yellow
Dimethylamino-benzaldehyde R.	—	+	++	++	++	—	—	[3] Violet
Lead acetate R. (cysteine)	—	—	—	+++	++	—	—	[4] Purple
Ninhydrin (amino acids)	+[1]	+++[2]	+++[3]	+++[4]	++[1]	—	+	[5] Precipitate
Biuret reaction	+	+	+	++	+++	—	—	[6] Brownish
Xanthoproteic reaction	+	++	++	+++[5]	++	+	—	[7] Greenish (ketoaldonic acids)
Molisch test (polysaccharide)	+[2]	+[3]	+[2]	+[3]	++[6]	+[3]	++[7]	
Reducing carbohydrate (triphenyltetrazolium chloride)	—	+++	+	+	++	+++	++	— No reaction
Bial's test (pentoses)	—	—	—	—	—	—	—	+ Slight reaction
Kiliani test (deoxy-sugars)	—	—	—	—	—	—	+	++ Strong reac-+++ tion

NOTE: R. = reagent.

TABLE IV

Simultaneous titration of trial bleedings from rabbit No. 1600 against alkaline saline extract of *T. spiralis* larvae

Weeks after infection	Serum 1 in: 8	16	32	64	128	256
1	—	—	—	—		
2	—	—	—	—		
3	—	—	—	—		
5	++++	++++	+++	++	—	
6	++++	++++	++++	++++	+++	—
7	++++	++++	++++	++++	+++	—

NOTE: Antigen was diluted to contain two antigenic units.

samples from the rabbit No. 1600 were titrated against two units of this antigen. The results tabulated in Table IV show that with this antigen antibodies are not demonstrable during the first 3 weeks. The maximum titer developed 6 weeks after infestation.

Discussion

The present investigation revealed the complexity of antigenic organization of *T. spiralis*. Seven antigenic fractions have been isolated and some proof has been obtained, either by serology or ultraviolet absorption spectra, that they are distinct. Their antigenicity was further demonstrated by production of allergic reactions in the skin of a *Trichinella*-infested rabbit.

It is of importance to note that the circulating antibodies against various fractions, at least in the rabbit, appear at different times during the course of infection. This may well explain, at least in part, the inconsistent results obtained with the complement fixation test by different workers (Frisch *et al.* (2, 3) and Tompkins (8)) who have observed that in some cases no antibodies were demonstrable at all, while in other instances the appearance of complement-fixing antibodies was detectable only late in the course of illness. In view of the results reported here, the possible explanation of such observations may lie in the type of the antigens used by various investigators. Saline-extracted antigen, for instance, as our results indicate, does not appear suitable for the early detection of antibodies, at least in experimental animals. The success of serological diagnosis by means of complement fixation test would seem, therefore, to depend on the choice of a proper antigen. Fraction V, described here, appears to be the solution to this problem, since the antibodies for this fraction, at least in experimental animals, appear early in the course of infection and remain at a high titer throughout.

Our experience with human sera, so far, is very limited but the results we have obtained are very encouraging. The antigen fraction V displayed a high degree of sensitivity as well as specificity regardless of the stage of illness at which a particular sample of serum was collected. Some of the sera tested were kindly supplied by Dr. H. Suessenguth, Mount Sinai Hospital, Cleveland.

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SPECIFICITY OF THE CLEARANCE PHENOMENON IN THE LUNGS OF PERTUSSIS-IMMUNIZED MICE FOLLOWING INTRATRACHEAL ADMINISTRATION OF RADIOIODINATED PERTUSSIS VACCINE[1]

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Abstract

In mice which were immunized with various immunizing agents 6 and 20 days prior to challenge, it was found that stimulation of lung clearance of intratracheally administered radioiodinated pertussis vaccine was observed chiefly in those mice which had received vaccine prepared from any of the three members of the genus *Bordetella*. The most marked effect occurred in the pertussis-immunized animals. In pertussis-immunized mice which were challenged intratracheally with heterologous radioiodinated antigens, the most significant increases in clearance were obtained in those animals receiving labeled *Bordetella* vaccines. When mice immunized with the various antigens were challenged by their labeled homologous antigens, again the most significant clearance stimulations occurred in the mice immunized and challenged with the *Bordetella* preparations. Clearance studies with mice immunized and/or challenged with a vaccine prepared from phase IV pertussis organisms would support a view that somatic antigens may stimulate lung clearance in mice immunized 6 days prior to challenge whereas the surface antigens may provoke a slower-developing and longer-lasting immunity that is responsible for the faster clearance observed 20 days after immunization.

Introduction

In previous work from this laboratory (18), it was shown that mice receiving a single immunizing dose of pertussis vaccine 1 or more days prior to the intratracheal administration of radioiodinated pertussis vaccine (I^{131}-PV) were able to remove a significantly greater amount of radioactivity from their lungs than non-immunized mice. It was suggested that the faster clearance in mice challenged 9 days or longer after immunization could be due to increased phagocytosis enhanced by specific antibodies. However, this assumption did not appear valid for the mice immunized 1 or 6 days before challenge since we were not able to detect *B. pertussis* antibodies in their sera. Evans and Perkins (9) described an interference type of immunity present in mice within a few hours after immunization with pertussis vaccine which they showed reached its maximum effect in about 10 days and then disappeared. Andersen (3), on the other hand, quoting the work of Nunes (21), in which antibodies to pneumococci were demonstrated in the sera of guinea pigs as early as 5 hours after intraperitoneal injection of the organisms, suggested this effect could be due to local antibody formation. The specificity of this early clearance phenomenon from mouse lung thus became of interest and our attempts to throw some light on this reaction form the basis of this report.

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Contribution from the Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

Methods

Mice

White mice, 19 to 27 g in weight, were used. Animals of the same sex and of similar age and weight as those immunized were used as the non-immunized controls.

Challenge Procedure

Mice were challenged by the intratracheal route as described previously (18).

Removal of Tissues

At the time of killing, the mice were bled and the lungs removed, cleaned of adhering tissue, and placed on planchettes for radioactivity measurements.

Radioactivity Measurements

Measurements of radioactivity were made using a scintillation counter as described previously (18).

Immunization

Mice in groups of approximately 20 were each given a single intraperitoneal injection (unless otherwise indicated) of the immunizing agent. This was followed by intratracheal challenge with I^{131}-PV or the radioiodinated homologous antigen after a period of either 6 days or 20 days. The immunizing agents and the doses administered were as follows:

1. Pertussis vaccine (preparation from phase I organisms)—(PV)—0.5 ml of a suspension containing approximately 2.4×10^9 organisms/ml.
2. Bovine serum albumin (BSA)—0.5 ml of a saline solution containing 12.5 mg albumin/ml.
3. Typhoid–paratyphoid A and B vaccine—0.5 ml of a 1:2 dilution containing *S. typhi*, 750×10^6/ml; *S. paratyphi* A, 250×10^6/ml; *S. paratyphi* B, 250×10^6/ml.
4. *B. bronchisepticus* vaccine—0.5 ml of a suspension of equivalent turbidity to the phase I pertussis vaccine (turbidity checked spectrophotometrically).
5. *B. parapertussis* vaccine—0.5 ml of a suspension of equivalent turbidity to the phase I pertussis vaccine.
6. *B. abortus* vaccine—0.5 ml of a suspension of equivalent turbidity to the phase I pertussis vaccine.
7. Diptheria toxoid—0.5 ml undiluted.
8. BCG vaccine—0.1 ml containing approximately 3.46×10^6 organisms. (This vaccine was administered subcutaneously.)
9. Sheep erythrocytes—0.5 ml of a suspension of 25 ml packed cells made up to 100 ml with physiological saline.
10. Pertussis vaccine (preparation from phase IV organisms)—(PV-phase IV)—0.5 ml of a suspension of equivalent turbidity to the phase I pertussis vaccine.

Preparation of Challenge Agents

The typhoid, bronchisepticus, parapertussis, and the phase IV pertussis vaccines, which were used in experiments to determine specificity of the reaction, were radioiodinated by a technique described elsewhere (17).

The bovine serum albumin (BSA) was iodinated in ammoniacal medium by the trace-labeling method of Francis, Mulligan, and Wormall (10) using a potassium iodide–potassium iodate system to which sodium iodide–I^{131} had been added. Iodine was liberated in this system by the addition of hydrochloric acid and the solution was added dropwise to the albumin with continuous stirring over a period of 15 minutes. Carrier sodium iodide was then added and the protein solution stirred for another minute. Removal of free iodine was carried out by McFarlane's technique (19) using an Amberlite IR 4B anion-exchange resin.

Results

The results of our experiments to demonstrate the effect of various immunizing agents on the clearance of I^{131}-PV from the lungs of mice are shown in Table I. Twenty-four hours after challenge with I^{131}-PV, a significantly greater clearance of radioactivity from the lungs was noted in mice immunized 6 and 20 days earlier with phase I pertussis vaccine than that noted in the control non-immunized mice. Similarly, significantly faster clearance rates were obtained with mice immunized with bronchisepticus vaccine 6 days before challenge and with BSA and parapertussis vaccine given 20 days

TABLE I

Effect of various immunizing agents on the clearance of radioiodinated pertussis vaccine from the lungs of mice at 24 hours following its intratracheal administration

Immunizing agent	Days of immunization	Percentage of administered dose ± S.E.M.		*P* values
		Immunized	Non-immunized	
PV*	6	15.5±0.39 (19)†	19.7±0.75 (25)	$P < 0.001$
PV	20	14.2±0.44 (87)	16.9±0.53 (70)	$P < 0.001$
BSA	20	15.8±0.42 (106)	18.9±0.35 (89)	$P < 0.001$
Bronchisepticus	6	17.7±0.47 (45)	19.7±0.53 (41)	$P < 0.01$
Parapertussis	20	14.6±0.50 (43)	17.1±0.56 (46)	$P < 0.01$
Parapertussis	6	14.6±0.53 (20)	16.6±0.64 (21)	$P < 0.05$
PV-phase IV	6	17.5±0.68 (40)	19.5±0.64 (46)	$P < 0.05$
Abortus	6	17.4±0.69 (21)	19.3±0.74 (24)	$P > 0.05$
Typhoid	20	17.6±0.63 (45)	19.3±0.80 (23)	$P = 0.1$
BSA	6	19.5±0.89 (16)	19.7±0.75 (25)	$P > 0.8$
BCG	20	13.4±0.60 (28)	13.2±0.48 (29)	$P > 0.7$
Abortus	20	19.9±0.90 (26)	19.3±0.74 (24)	$P > 0.6$
Bronchisepticus	20	21.1±0.73 (39)	20.7±0.53 (43)	$P > 0.6$
Diphtheria	20	19.5±1.21 (19)	17.8±0.80 (19)	$P > 0.2$
Typhoid	6	14.3±0.54 (29)	13.2±0.48 (29)	$P > 0.1$
Sheep RBC	20	14.2±0.59 (29)	13.2±0.48 (29)	$P > 0.1$
PV-phase IV	20	21.3±0.58 (47)	18.9±0.52 (43)	$P < 0.01$‡

*Immunizing agents listed in descending order with respect to their stimulation of clearance of I^{131}-PV.
†Figures in parentheses denote the number of mice.
‡Retarded clearance.

TABLE II

The clearance of heterologous radioiodinated antigens by the lungs of pertussis-immunized mice at 24 hours following their intratracheal administration

Challenge antigen	Immunizing agent	Percentage of administered dose ± S.E.M.		P values
		Immunized	Non-immunized	
I^{131}-bronchisepticus	PV-6*	27.1±0.88 (20)†	32.3±0.95 (13)	P <0.001
	PV-20	25.7±0.90 (12)	31.6±1.55 (21)	P <0.01
I^{131}-parapertussis	PV-6	10.0±0.49 (23)	13.9±0.79 (20)	P <0.001
	PV-20	14.1±0.60 (22)	16.1±0.79 (25)	P <0.05
I^{131}-PV-phase IV	PV-6	51.2±1.33 (20)	55.8±0.94 (20)	P <0.01
	PV-20	47.4±2.04 (7)	43.7±0.95 (22)	P >0.1
I^{131}-typhoid	PV-6	20.5±0.55 (15)	20.1±0.73 (17)	P >0.6
	PV-20	16.3±0.42 (31)	18.7±0.43 (37)	P <0.001
I^{131}-BSA	PV-6	24.2±0.82 (22)	25.9±1.34 (22)	P >0.2
	PV-20	24.7±1.28 (19)	24.2±1.79 (20)	P >0.8

*Immunizing agent followed by the number of days of immunization.
†Figures in parentheses denote the number of mice.

before challenge. Faster clearance, but of a borderline significance, was also noted in mice immunized with either parapertussis vaccine or phase IV pertussis vaccine 6 days earlier. On the other hand, a retarded clearance was obtained with mice immunized with the latter preparation 20 days before challenge.

Table II shows the results of challenging mice immunized 6 and 20 days earlier with phase I pertussis vaccine and non-immunized mice with radioiodinated typhoid, bronchisepticus, parapertussis, and phase IV pertussis vaccines as well as with radioiodinated BSA. A significantly faster clearance of labeled bronchisepticus, parapertussis, and phase IV pertussis vaccines was obtained with mice immunized 6 days earlier than with the non-immunized controls. In the mice immunized 20 days before challenge, however, this clearance rate, while still significant, was not as great for the labeled bronchisepticus and parapertussis organisms, and there was no significant difference between the immunized and non-immunized mice in the clearance rate of iodinated phase IV pertussis organisms. On the other hand, radioiodinated typhoid vaccine was cleared more rapidly in the mice immunized 20 days before challenge with phase I pertussis vaccine than in the controls. No such difference was observed between mice immunized only 6 days before challenge with phase I pertussis vaccine and the controls. The I^{131}-labeled BSA was not cleared from the lungs of the mice immunized either 6 days or 20 days before challenge with phase I pertussis vaccine faster than from the controls. It may also be noted that the level of radioactivity in the lungs of mice challenged with phase IV I^{131}-PV was much higher than in the mice challenged with the other radioiodinated antigens.

It was shown in Table I that mice immunized either 6 or 20 days earlier with phase I pertussis vaccine could clear more I^{131}-PV from their lungs in 24 hours than non-immunized mice. Table III shows the capacity of mice

TABLE III

Comparison of PV with other immunizing antigens in the lung clearance of their homologous radioiodinated antigens

Challenge antigen	Immunizing agent	Percentage of administered dose ± S.E.M.		P values
		Immunized	Non-immunized	
I^{131}-PV	PV-6*	15.5±0.39 (19)†	19.7±0.75 (25)	P <0.001
	PV-20	14.2±0.44 (87)	16.9±0.53 (70)	P <0.001
I^{131}-bronchisepticus	Bronchisepticus-6	22.9±0.75 (16)	32.3±0.95 (13)	P <0.001
	Bronchisepticus-20	24.0±0.98 (21)	31.6±1.55 (21)	P <0.001
I^{131}-parapertussis	Parapertussis-6	10.0±0.47 (13)	13.9±0.79 (20)	P <0.001
	Parapertussis-20	12.3±0.84 (16)	16.1±0.79 (25)	P <0.01
I^{131}-PV-phase IV	PV-phase IV-6	48.1±1.57 (19)	55.8±0.94 (20)	P <0.001
	PV-phase IV-20	42.4±1.00 (22)	43.7±0.95 (22)	P >0.3
I^{131}-BSA	BSA-6	25.1±1.19 (21)	25.9±1.34 (22)	P >0.6
	BSA-20	26.2±1.89 (15)	24.2±1.79 (20)	P >0.4
I^{131}-typhoid	Typhoid-6	22.7±0.62 (12)	20.1±0.73 (17)	P <0.02
	Typhoid-20	16.7±0.79 (17)	17.7±0.64 (15)	P >0.3

*Immunizing agent followed by the number of days of immunization.
†Figures in parentheses denote the number of mice.

TABLE IV

Statistical evaluation of the effects of various immunizing agents on the stimulation of lung clearance of radioiodinated antigens in mice

Immunizing agent	Radioiodinated challenge antigen					
	I^{131}-PV	I^{131}-bronchisepticus	I^{131}-parapertussis	I^{131}-PV-phase IV	I^{131}-BSA	I^{131}-typhoid
PV-6	P <0.001	P <0.001	P <0.001	P <0.01	P >0.2	P >0.6
PV-20	P <0.001	P <0.001	P <0.05	P >0.1	P >0.8	P <0.001
Bronchisepticus-6	P <0.01	P <0.001				
Bronchisepticus-20	P >0.6	P <0.001				
Parapertussis-6	P <0.05		P <0.001			
Parapertussis-20	P <0.01		P <0.01			
PV-phase IV-6	P <0.05			P <0.001		
PV-phase IV-20	P <0.01*			P >0.3		
BSA-6	P >0.8				P >0.6	
BSA-20	P <0.001				P >0.4	
Typhoid-6	P >0.1					P <0.02*
Typhoid-20	P =0.1					P <0.3

*Retarded clearance.

immunized with other antigens, viz., BSA, typhoid, bronchisepticus, parapertussis, and phase IV pertussis vaccines, to clear their lungs of the homologous radioiodinated antigens, and it shows as well their ability to clear I^{131}-PV. Both groups of the bronchisepticus- and parapertussis-immunized mice showed significantly greater clearance of their respective labeled antigens than did the non-immunized mice. The phase IV pertussis-immunized mice showed this effect only in the group immunized 6 days before challenge. The clearance rate of radioactive BSA was the same in both immunized and non-immunized animals, and a retarded clearance of radioiodinated typhoid vaccine was observed in the mice immunized with typhoid vaccine 6 days earlier.

Table IV is a summary of the pertinent data contained in Tables I, II, and III and shows the levels of statistical significance of the results between immunized and non-immunized mice.

Discussion

The present study was designed to determine the specificity of the clearance of I^{131}-labeled pertussis organisms from the lungs of mice. Animals were immunized with various antigens and challenged intratracheally after 6 or 20 days with radioiodinated pertussis vaccine. The clearance of this radioactive material was measured by counts taken on lungs removed from the mice 24 hours after the challenge. Three antigens other than phase I pertussis vaccine produced this rapid clearance effect. These were BSA, bronchisepticus vaccine, and parapertussis vaccine. This rapid clearance by animals immunized with bronchisepticus vaccine and parapertussis vaccine is understandable since it has been well established (1, 2, 4, 6, 7, 8, 15) that these organisms have antigens common to pertussis vaccine, and both produce toxins identical with that produced by *B. pertussis*. This close relationship in antigenic structure was further confirmed by the increased clearance of both radioiodinated bronchisepticus vaccine and radioiodinated parapertussis vaccine by phase I pertussis-immunized mice. We have no explanation for the effect produced by the BSA other than that it was a chance result. Notwithstanding the statistical results with six pooled experiments, a significant clearance was not obtained in three out of these six experiments. Conversely, the phase I pertussis-immunized animals did not accelerate clearance of I^{131}-labeled BSA, but since the mice immunized with BSA also failed to clear this antigen there is a possibility that the I^{131}-BSA was denatured in the iodination process.

The converse experiment of challenging phase I pertussis-immunized mice with other radioiodinated antigens was carried out to establish whether a cross clearance effect existed among those antigens capable of stimulating clearance of I^{131}-PV. This was found to be true in each case with the exception of the I^{131}-labeled BSA mentioned above. There was marked accelerated clearance of radioiodinated bronchisepticus, parapertussis, and phase IV pertussis vaccines in phase I PV-immunized mice. The clearance of I^{131}-PV by bronchisepticus, parapertussis, and phase IV PV-immunized mice was not nearly as great. In addition, the clearance of radioiodinated typhoid vaccine was notably increased in the mice immunized with phase I pertussis vaccine 20 days before challenge. The reason for this is obscure particularly since the clearance values of radioactive typhoid organisms were no higher than the controls in the typhoid-immunized mice.

Mice immunized with antigens other than phase I pertussis vaccine were also challenged with their homologous radioiodinated derivatives to determine whether the rate of lung clearance in these animals was comparable to that observed in the phase I pertussis-immunized mice. The comparably high clearance rates observed with bronchisepticus- and parapertussis-immunized mice are probably attributable to the antigenic similarity of these vaccines to pertussis vaccine. It was found that in mice immunized with phase IV pertussis vaccine, only the group immunized 6 days before challenge cleared the homologous radioiodinated antigen faster than the controls. These mice were also able to accelerate the clearance of I^{131}-PV. Similarly, I^{131}-labeled

phase IV pertussis organisms were cleared faster than in the controls only in the mice immunized with phase I pertussis vaccine 6 days earlier. It is rather interesting that this stimulated clearance was not evident in animals immunized 20 days before challenge and, in fact, in this group clearance of I^{131}-PV was actually retarded. Phase IV *B. pertussis* organisms are generally considered to lack the surface antigens found in phase I. On this assumption, it may be deduced from our results that the presence of these surface antigens is necessary in both the immunizing and challenging agents in order to stimulate the clearance in mice immunized 20 days before challenge. If this be true, then it might also be concluded that the positive clearance results in the mice immunized 6 days earlier could be due to common somatic antigens in the immunizing and challenging agents. The retarded clearance in mice immunized 6 days before challenge with typhoid vaccine and the insignificant clearance effect in those immunized 20 days before challenge is of interest. In our laboratory, Tolnai (22) has found that peak immunization of mice by T.A.B. vaccines occurs about the 6th day. However, any relationship our findings might bear to the protective capacity of these vaccines in mice has not been investigated.

There are a number of reports (5, 12, 13, 16) in the literature concerning the removal of labeled antigens from the blood stream of normal and specifically immunized animals. It is generally found that the immune animal is capable of clearing the labeled material from its blood stream much more rapidly than the non-immunized one. Francis, Mulligan, and Wormall (11) have stated that the rate at which an antigenic protein is removed from the blood stream is, to some extent, an index of the "immune response" of the animal to that protein. However, very little work seems to have been reported in the literature regarding the removal of labeled antigens from the lung. Halpern and Liacopoulos (14) have studied the removal of I^{131}-ovalbumin and bovine serum albumin from the lungs of guinea pigs but this work has been carried out mainly using non-immunized animals. They found that the I^{131}-BSA had been cleared from the lungs in 3 days and that the residual labeled BSA in the plasma suddenly disappeared at the 9th day as a result of the appearance of antibody at that time. On the other hand, with animals which were challenged intravenously and which had received an injection of BSA 20 days earlier, plasma clearance was rapid and complete in 4 to 5 days due to the presence of antibody. As pointed out in our earlier paper (18), the faster clearance of I^{131}-PV by pertussis-immunized mice takes place prior to the appearance of measurable circulating antibodies. The formation of local tissue antibody in the respiratory tract has been considered as a possibility in intranasal immunization (20) and it is at least possible that such a local immunity developed in the intraperitoneally immunized mice and was responsible, in part, for the faster clearance of I^{131}-PV in mice immunized for shorter periods. If such is the case, it would be expected that intratracheal immunization would be more effective than intraperitoneal immunization in enhancing clearance. Experiments in progress would indicate that this is probably true.

From these studies, it would appear that the specificity in the clearance of I^{131}-labeled pertussis vaccine from mouse lungs, after intratracheal administration, is not absolute. Increased clearance was noted not only in phase I pertussis-immunized mice but also in mice immunized with two other members of the genus *Bordetella*. There is, in these results, the suggestion that the faster clearance of I^{131}-PV noted in the mice challenged 6 days after immunization with pertussis vaccine may be due to the somatic antigens whereas the faster clearance which shows up in the mice challenged 20 days after immunization is due to the surface antigens which produce a longer-lasting but later-appearing immunity. Further support for this view has been obtained in fractionation studies of pertussis vaccine recently carried out in our laboratory.

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NOTES

OXIDATION OF LABELLED SUBSTRATES BY DWARF BUNT, TILLETIA CONTRAVERSA[1]

R. W. NEWBURGH AND VERNON H. CHELDELIN[2]

The use of specifically labelled radioactive substrates makes it possible to determine the relative contribution of pathways to the metabolism of an organism. The radiorespirometer provides the tool by which such comparisons can be made (2). Briefly, this consists of a reaction flask mounted on a standard Warburg manometer and connected to a CO_2 trap. A continuous flow of gas sweeps the CO_2 (containing $C^{14}O_2$ obtained from the oxidation of a labelled substrate) from the reaction flask into the trap containing sodium hydroxide. This solution can then be removed at desirable time intervals and the $C^{14}O_2$ precipitated as $BaC^{14}O_3$, plated, and counted by the usual procedures. Previous studies showed that there was a difference in glucose oxidation by the mycelium and the teliospores of the wheat smut fungus, *Tilletia caries*, race T-10 (1). The Embden–Meyerhof pathway accounted for 66% of the glucose oxidation and the pentose cycle 34% when mycelia were used while glucose was oxidized exclusively by the Entner–Doudoroff pathway by teliospores. The present report indicates that a difference in catabolism also occurs between teliospores of wheat smut fungus and of dwarf bunt. Since the dwarf bunt has not been grown in artificial culture, a comparison could not be made between mycelia of this organism and that of wheat smut fungus.

The procedures used were the same as reported previously (1). Figure 1 shows the utilization of glucose by dwarf bunt spores. Calculation of the per cent participation by the pentose cycle and of the Embden–Meyerhof pathway by previous procedures (1) shows that 67% of the glucose was oxidized by the pentose cycle and 33% by the Embden–Meyerhof pathway. Contrary to results with spores of *T. caries* race T-10, none of the glucose is oxidized by the Entner–Doudoroff pathway since the recovery of $C^{14}O_2$ from C-4 of glucose was 92% (by calculation) compared with 69% from C-1 of glucose. If this pathway were operative in dwarf bunt, the yield of $C^{14}O_2$ from C-1 should equal or exceed that from C-4.

Several other labelled substrates besides glucose were tested, viz. glutamic acid-1-C^{14}, ribose-1-C^{14}, and Δ-gluconolactone-1-C^{14}. The results are shown in Fig. 2. Little oxidation of substrates other than glucose occurred. Since

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[2]Respectively, Crop Research Division, Agricultural Research Service, U.S. Department of Agriculture; and Science Research Institute, Oregon State College, Corvallis.

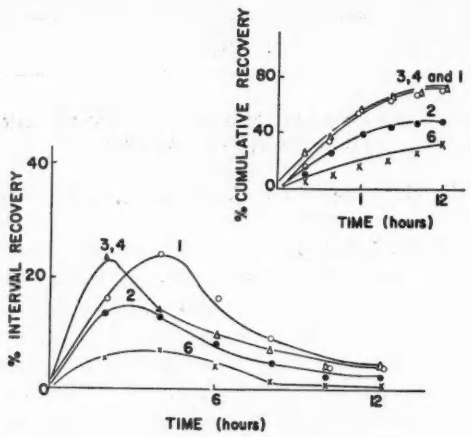

FIG. 1. Recovery of $C^{14}O_2$ from labelled glucose by dwarf bunt spores. Each flask contained 1.5 ml of cell suspension (450 mg of spores), 0.5 ml of 0.2 *M* phosphate buffer, pH 6.5, 13 μM of substrate containing 0.3 μc of radioactive material as indicated. 1 = Glucose-1-C^{14}; 2 = glucose-2-C^{14}; 6 = glucose-6-C^{14}; 3,4 = glucose-3,4-C^{14}.

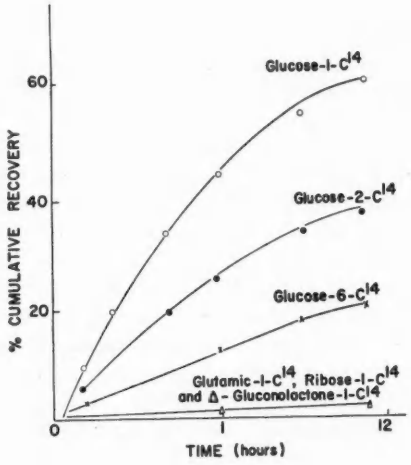

FIG. 2. Recovery of $C^{14}O_2$ from labelled substrates by dwarf bunt spores. Additions same as in Fig. 1.

TABLE I

Effects of pH on rate of oxidation to $C^{14}O_2$ of various C^{14}-labelled substrates by dwarf bunt spores (additions same as listed for Fig. 1)

Substrate	pH					
	4.0	5.0	6.0	6.5	7.0	7.5
	Radioactivity in collected $C^{14}O_2$, counts/minute/hour					
Glutamic-1-C^{14}	231	220	353	264	—	336
Ribose-1-C^{14}	22	13	25	404	300	259
△-Gluconolactone-1-C^{14}	45	41	191	197	680	211
Acetate-1-C^{14}	1545	1543	1830		1722	
Acetate-2-C^{14}	567	659	909		646	
Glucose-1-C^{14}	2000	2231	2251	2180	2260	2280
Glucose-2-C^{14}	1583	1555	1400	1580	1530	1510
Glucose-6-C^{14}	1062	1117	1203	830	880	660

it is possible that permeability could be a factor in the oxidation of these substrates, the effect of pH was studied. It appears that little effect of pH could be noted except with ribose and △-gluconolactone (Table I). The effect of pH upon these two substrates may reflect improved kinase activities at pH 6 to 7, which bring about the necessary phosphorylations prior to oxidation of each compound. Although the results indicate the presence of the pentose cycle and tricarboxylic acid cycle, it appears that several substrates known to be oxidized by this complex of enzymes are not readily utilized. The utilization of acetate-1-C^{14} and acetate-2-C^{14} to a greater extent than glutamic-1-C^{14} is of interest. Although one cannot exclude permeability, possibly the conversion of glutamic acid to α-ketoglutarate in this organism is sluggish and therefore this metabolite is not readily oxidized via the tricarboxylic acid cycle. Alternatively, the glutamic acid might be used more extensively for other processes and thus only small amounts are available to reactions leading to oxidation.

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THE EFFECT OF HOMOGENIZING THE INOCULUM ON IN VITRO PRODUCTION OF ERGOT ALKALOIDS[1]

W. A. TABER AND L. C. VINING

Homogenizing (blending) the vegetative inoculum was reported previously (3) to result in an increase in the production of alkaloids by stationary cultures of *Claviceps purpurea* (PRL 1578) during growth periods of up to 35 days.

The effect of varying the blending time on the yield of alkaloid has now been determined by homogenizing a single standard secondary culture, grown in YD broth, for various intervals and using aliquots to inoculate flasks of the production medium, 10-B-galactose (see (3) for details). An optimal blending period clearly existed (Table I). The decreased yield with 60 seconds' blending is probably not due to the effect of heat on the inoculum, since the temperature of the inoculum during blending did not rise above 29° C, therefore the data lend support to the claim (1) that extended blending is harmful. The view that all but the briefest possible blending times are harmful (1) is not supported by these data, however, as there was no appreciable decrease in yield of alkaloid when the blending time was extended from 5 seconds to 30 seconds. Further, 10 seconds' blending resulted in an increased rate both of alkaloid production and of growth (Fig. 1). The increased growth rate of cultures started with blended inocula is presumably due to an increase in the number of potential centers of growth, and to the fact that the dispersed and air-charged fragments of mycelium remained near the surface of the medium. It has been observed that cultures started with blended inocula formed a dry-surfaced mat by approximately 10 days, while cultures started with unblended inocula required approximately 30–35 days for dry mat formation. The difference in behavior of the two forms of inocula was noticeable from the first. The dense, unblended inoculum settled to the bottom of the medium within 1 hour of inoculation, and then slowly developed into a submerged mat, which, through folding and buckling during growth, eventually reached the surface. The less-dense, blended inoculum rose to the surface within 1 hour of inoculation, and then grew at the surface, forming a continuous, but still partially submerged, mat by 4 days. The differences observed between the cultures started with homogenized and with unhomogenized inocula are probably related to oxygen supply although the effects of oxygen on growth have not been studied.

Blending apparently accelerated activities other than growth. The efficiency with which sugar was converted to cellular carbon (economic coefficient (1)) by cultures started with blended inocula was lower than that in cultures started with unblended inocula (Figs. 1 and 2). This diversion of nutrient from growth activity may be at least partially accounted for by the greater amount of ergot alkaloid and organic nitrogen released into the medium

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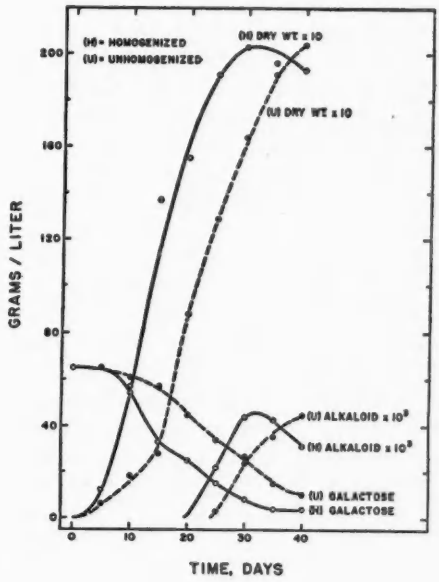

FIG. 1. The course of growth, sugar utilization, and alkaloid production by cultures started with mycelia homogenized 10 seconds and mycelia that was not homogenized.

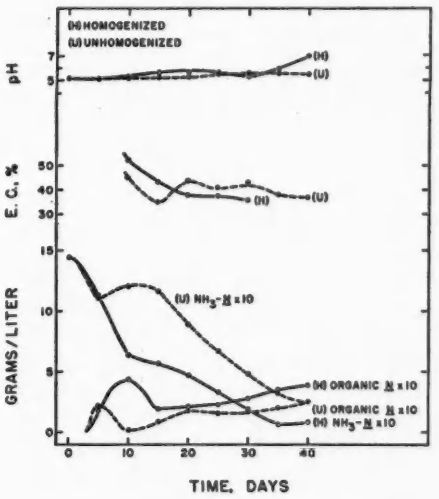

FIG. 2. The course of ammonia utilization, release of organic nitrogen, and pH of cultures described in Fig. 1. E.C. refers to the economic coefficient for each time interval.

TABLE I

PRL 1578
Effect of homogenizing period on yield of alkaloid

Time of homogenizing, seconds	Incubation period, days			
	23	30	35	44
	Milligrams alkaloid/liter*			
None	0.6 ± 0.1	21.0 ± 1.4	39.1 ± 2.7	32.1 ± 3.2
5	24.1 ± 0.5	55.9 ± 7.7	58.4 ± 0.6	42.2 ± 3.9
30	24.8 ± 3.8	51.7 ± 10.0	55.9 ± 6.4	44.1 ± 3.2
60	18.4 ± 4.2	32.6 ± 3.4	41.4 ± 10.2	43.8 ± 3.8

Inoculum: Unhomogenized — equivalent to 5.1 mg dried mycelium/flask,
5 seconds — equivalent to 2.6 mg dried mycelium/flask,
30 seconds — equivalent to 2.7 mg dried mycelium/flask,
60 seconds — equivalent to 2.9 mg dried mycelium/flask.
*The method of analysis is described in (3).

TABLE II

PRL 1578
Effect of various quantities of homogenized mycelia on yield of alkaloid

Inoculum size, mg/flask*	Treatment	Incubation period, days		
		25	30	45
		Milligrams alkaloid/liter		
13	Homogenized	27.8 ± 1.5	70.2 ± 2.7	76.0 ± 2.4
13	Unhomogenized	2.4 ± 1.0	30.3 ± 8.0	94.5 ± 0.1
5	Homogenized	19.7 ± 1.2	61.5 ± 4.1	76.4 ± 3.0
5	Unhomogenized	Nil	15.0 ± 2.5	56.5 ± 7.9
0.5	Homogenized	0.6 ± 0.1	12.1 ± 1.4	50.0 ± 8.0
0.5	Unhomogenized	Nil	Nil	13.3 ± 14.7

Analysis: All pairs of homogenized and unhomogenized values differ significantly ($P < .05$).
*Based on dry weight of mycelium.

during the early growth period. The very high economic coefficient of all cultures during the early incubation period is undoubtedly due to the preferential utilization of succinic acid as a carbon source.

To examine the effect of inoculum size with blended and unblended mycelia upon both the total yield and the time required to achieve detectable yield, aliquots of mycelial suspensions of various concentrations were added to one set of flasks containing the production medium. The remainder of each suspension was blended 10 seconds and aliquots added to the second set of production flasks. These results (Table II) again demonstrate that blending accelerates the rate of production at all concentrations of inoculum. Combinations of a heavy inoculum and blending favored rapid production and gave high alkaloid levels in the shortest possible time, but the highest total yield was obtained through long incubation of cultures started with heavy, unblended inocula. Clearly the effect of blending cannot be duplicated by merely increasing the concentration of the inoculum, and in this

respect the results differ from those obtained for penicillin production by Savage and Vander Brook (2). With very dilute, blended inocula these authors were able to achieve yields equal to the maximum obtained for a less diluted, unblended inoculum at the same time interval. Peak production was greater with cultures started with a diluted, blended inoculum and was reached after a longer growth period. However, it must be noted that the experiments on penicillin production were carried out with shaken cultures where the added effects of aeration and mat formation, noted above, would not be expected to operate.

In summary, under the conditions of growth used, the effect of blending the inoculum is to induce more rapid but less efficient growth of the organism and correspondingly rapid, but not necessarily maximal, alkaloid production. The promotion of early growth at the surface of the medium was also noted.

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A MODIFIED MEMBRANE FILTER CULTURE PROCEDURE AND ITS USE FOR ASSESSMENT OF NUTRIENT IN AGAR

J. J. MILLER

In the usual cultural methods employing large inocula little or no growth is observed on agar without added nutrients, but with small inocula an appreciable cell multiplication may occur. Ryan (1) found that cells of *Escherichia coli* produced microcolonies on agar in the absence of an added assimilable carbon source. Careful washing of the agar reduced the size of the colonies. In an attempt to develop a method for studying sporulation of *Saccharomyces cerevisiae* on pieces of millipore membrane filter placed on various moist substrata it was found that commercial agar could not be used because the cells multiplied considerably, and, as Saito (2) has indicated, growth and sporulation in this organism seem mutually exclusive. The possibility that the method might be of value for assessing the freedom of agar from nutrients able to support growth of microorganisms was suggested to the writer by Dr. W. Yaphe, who supplied a sample of agar that had been washed continuously in 0.1% Versene until the effluent was free of

carbohydrate (3) for comparison with commercial agar in this respect. The following is a description of the method, which involves exposing a small number of cells to a comparatively large volume of substratum in a manner that allows convenient determination of the amount of growth produced by each cell of the inoculum.

Millipore membrane filter of type TV 20 μ thick with pore size 50 $\mu\mu$, is cut into pieces 5×10 mm which are sterilized by autoclaving in distilled water, and placed on the surface of sterilized filter paper in a petri dish. Each piece is seeded with a platinum loop of washed cell suspension. If the petri dish contains two 9-cm Whatman No. 3 filter papers with one 7-cm No. 1 filter paper on top, the whole being moistened with 5 ml sterilized water or buffer, the suspension liquid is drawn through the membrane into the filter papers by surface tension forces within a few seconds, leaving the cells on the surface. (This operation makes a filtration funnel unnecessary for membrane seeding, with resulting economy in area of membrane needed per experiment.) The pieces of membrane may then be transferred conveniently with a small stainless steel spatula to any desired environment. At the conclusion of the period allowed for growth they are transferred to small drops of erythrosin-saturated lactophenol on a slide. The lactophenol partially clears the membrane and the cells are made more easily visible by the dye. The mounts thus prepared are semipermanent.

The usefulness of the method for nutritional research is indicated by two experiments undertaken to compare the growth of yeast in several environments usually considered of negligible nutrient content. In the first experiment 0.5-g portions of three agars were dissolved separately in 12.5-ml volumes of distilled water by autoclaving. The same volume of $M/20$ phthalate buffer of pH 5 was added to each and the mixtures were poured into petri dishes. A fourth petri dish contained one 7-cm No. 1 and two 9-cm No. 3 filter papers moistened with 5 ml $M/40$ buffer, and in a fifth with 12 ml buffer was placed a flamed Jenkins filtering block of unglazed porcelain 10 mm in diameter and 12 mm high. Pieces of membrane seeded with a 4-mm loop of suspension at a cell population density of 400,000 per ml were transferred to the dishes. After 3 days at 27° C the membranes were mounted in lactophenol, and growth on each substratum was estimated by inspection with the high dry objective of 50 randomly selected colonies. Buds less than half as large as the parent cell were not counted. Experiment 2 differed in that pH 5.5 buffer was used and each piece of membrane was seeded with a 3-mm loop of suspension at a cell population density of 100,000 per ml, which was found to give better dispersal of colonies. Cubes cut from a drying plate of unglazed porcelain 8 mm thick were substituted for the Jenkins filtering block. They were thoroughly washed and then heated in a crucible about 5 hours to drive off organic matter. Other experiments have shown that good growth occurs on membranes placed on such cubes when the latter are standing in a nutrient solution.

From the results shown in Table I it will be seen that the Versene-washed agar supported much less growth than the commercial agar. It supported

TABLE I

Multiplication of yeast cells on pieces of millipore membrane filter placed on several substrata without added nutrients

Environment	Average number of cells per colony after 3 days	
	Experiment 1	Experiment 2
Control*	2	2
Bacto-agar	100†	
Agar purified, Difco	100†	100†
Versene-washed agar	7	5
Filter papers‡	7	12
Jenkins filtering block	4	
Unglazed porcelain cube		2

*Membranes transferred to lactophenol slides directly after seeding.
†Rough estimates only, as it is not possible to see all cells in 3-dimensional colonies.
‡Moistened with 5 ml buffer in experiment 1, and 6 ml in experiment 2.

less growth than filter paper in experiment 2. In both experiments least growth occurred on porcelain. In fact, in the second experiment no increase over the control was detected. It would seem advisable, therefore, when using commercial agar as a growth substratum in investigating the nutritive value of a substance, to wash the agar thoroughly or else use large inocula. Other alternatives would be to use instead blocks of unglazed porcelain, or silica gel. It is unlikely, however, that this would eliminate "residual" growth in all cases since Ryan (1) traced the contaminating carbon source in his liquid cultures to an impurity in the inorganic salts.

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